RRP22 Is a Farnesylated, Nucleolar, Ras-Related Protein with Tumor Suppressor Potential

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Abstract

Ras proteins are members of a superfamily of related small GTPases. Some members, such as Ras, are oncogenic. However, other members seem to serve as tumor suppressors, such as Rig and Noey2. We now identify and characterize a novel member of the Ras superfamily, RRP22. Like Ras, RRP22 can be posttranslationally modified by farnesylation. Unlike Ras, RRP22 inhibits cell growth and promotes caspase-independent cell death. Examination of human tumor cells shows that RRP22 is frequently down-regulated due to promoter methylation. Moreover, reexpression of RRP22 in an RRP22-negative neural tumor cell line impairs its growth in soft agar. Unusually for a Ras-related protein, RRP22 localizes to the nucleolus in a GTP-dependent manner, suggesting a novel mechanism of action. Thus, we identify a new member of the Ras superfamily that can serve as a potential tumor suppressor. (Cancer Res 2005; 65(8): 3117-25)

Introduction

Ras proteins are part of a superfamily of related small GTPases whose activity is normally regulated by a GTP/GDP cycle (1). The members of this superfamily are involved in regulating a diverse array of biological processes. Some, such as the Ras proteins and some members of the Rho subfamily, exhibit oncogenic properties (2). Others, such as the Rig/Noey2 subfamily, exhibit the properties of tumor suppressors (3, 4). These biological extremes show the versatility of small G proteins in nature.

One of the defining characteristics of Ras proteins is that they undergo a posttranslational modification consisting of the addition of a farnesyl isoprenoid lipid to their COOH terminus (5). Farnesylation of Ras proteins is essential for their transforming functions. Without this modification, the proteins mislocalize in the cell (6) and exhibit reduced binding affinity for effectors (7, 8).

Because few other proteins undergo farnesylation, the enzyme responsible, farnesyl transferase, has been targeted for rational drug design (9). Several inhibitors of farnesyl transferase are currently in clinical development as cancer therapeutics. Ironically, it now seems that the biological effects of farnesyl transferase inhibitors are Ras independent (10). Thus, the true farnesylated target of farnesyl transferase inhibitors remains to be determined.

Using a bioinformatics approach, we identified a hypothetical gene, RRP22, encoding a novel protein with considerable homology to Ras and Rig. The RRP22 gene was first identified as one of the genes located at the chromosome 22, 12q site of frequent loss of heterozygosity (11), suggesting that RRP22 might play a role in tumorigenesis. Moreover, analysis of the predicted primary structure suggests that this protein may be one of the rare proteins that is modified by farnesylation. Consequently, it might play a role in the cellular response to farnesyl transferase inhibitors.

We now describe the cloning of the RRP22 cDNA and the biochemical and biological characterization of its protein product. We show that like Ras and Rig, RRP22 is a farnesylated protein. However, the biological effects of RRP22 are more akin to the Rig/Noey2 tumor suppressors (3, 4) than to the Ras oncoproteins.

Overexpression of RRP22 promotes cell cycle arrest and caspase-independent cell death. RRP22 is frequently inactivated by promoter methylation in human tumor cell lines. Furthermore, exogenous expression of RRP22 in a human neural tumor cell line impairs its growth in soft agar. Although the mechanism of action of RRP22 remains unknown, we show that RRP22 localizes to the nucleolus in a GTP-dependent manner. This is unusual for a Ras-related protein and may indicate a novel biological function in modulating transport of nucleolar components. Thus, we describe RRP22 as a novel, farnesylated, Ras-related protein with tumor suppressor potential that is implicated in nucleolar function.

Materials and Methods

DNA. RRP22 was identified by TBLASTn searches of the EST database using fragments of sequence from Rig (4). IMAGE clone 2166661 was ordered from the IMAGE Consortium and the gene isolated as a 5′–3′ fragment using oligomers 5′–GCCGGATCCATGGGGGG–TAGCTGCGGG–3′ forward and 5′–GGAGAITTCTCACACATGAGGCTG–GACGCCG–3′ reverse primers. RRP22 mutants were generated by PCR or Quickchange kit (Stratagene, La Jolla, CA). Clones were verified by sequence analysis before subcloning into pcDNA3.1 Flag (12), pZIP-NeoHABE (12), pBabe (13), pEGFP-C1 (Clontech, Palo Alto, CA), and pGEX2T (Pharmacia, Uppsala, Sweden). pCaspa3-3Sensor was purchased from Clontech. Green fluorescent protein-ADP-ribosylation factor (GFP-ARF) constructs were generous gifts of C. Sherr (St. Jude Childrens Research Hospital, Memphis, TN) and C. Asker (Karolinska Institute, Sweden).

Cell culture. Cells were grown in DMEM with 10% calf serum (NIH 3T3), 10% fetal bovine serum (HEK 293T, Cos-7), and 10% fetal bovine serum in RPMI 1640 (neural tumor cell lines). Transfections were done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Cell cycle assays were done as described previously (14).

Soft agar assays. T-98G human neural tumor cells were transfected with 50 ng of pBabe and 1 μg of either empty GFP vector or GFP-RRP22. Cells were selected in puromycin for 10 days, and 5,000 cells per well were then plated in soft agar as described previously. Colonies were scored under an inverted microscope after 4 weeks. Results shown are the average of two independent experiments done in triplicate.

Prenylation assays. Glutathione S-transferase (GST) fusion proteins were isolated as described previously (4). The recombinant proteins were then incubated in a rabbit reticulocyte lysate (Promega, Madison, WI) with

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and labeled with \( ^{32}P \)orthophosphate overnight. The cells were lysed and fractionated by centrifugation into a cytosol/plasma membrane supernatant and a nuclear pellet. The supernatant was further fractionated and the pelleted plasma membrane was resuspended in PBS. The nuclear pellet was resuspended in TSE buffer (15). Dounce homogenized, pelleted, and washed before being subjected to Western analysis.

**Apoptosis assays.** Apoptosis assays were done using the pCaspace-Sensor system (Clontech) as described previously (14) and terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assays using a Deadend kit (Promega).

**Promoter methylation assays.** Six glioma tumor cell lines (U87, A172, U343, U373, H4, and Hs683) and three normal brain tissues (N1, N2, and N3) were analyzed for promoter region methylation by combined bisulfite restriction analysis (CoBRA) and direct sequencing as described previously (16). The RRP22 CpG island was identified using CpG software (available at www.ebi.ac.uk) and is located at −490 > +1993 bp relative to the transcription start site of RRP22 (NM_006477) and an Obs/Exp of 1.07 and a CG percentage of 62.16. The promoter region was predicted using Sensor system (Clontech) as described previously (14) and terminal deoxycytidine (AzaC) treatment (RT-PCR) was done essentially as described previously (16). PCR primers were specific for exon 1 and exon 3 of RRP22 (NM_006477). Primers RRP22 CoBRA forward 5′-AGCGAGAGAGGATAGAGGCA-3′ and RRP22 CoBRA reverse 5′-CTCCCCRRCTAAAAACCTCATAAA-3′ were used. Sso1 artifically methylated normal blood DNA was used as a positive control. 5-Aza-2′-deoxycytidine (AzaC) treatment and expression analysis in glioma cell lines by reverse transcription–PCR (RT-PCR) was done essentially as described previously (16). PCR primers were specific for exon 1 5′-TTCCCTGCTGCTGACTACCC-3′ and exon 3 5′-CCCGTCCAGTTGTACTTGG-3′ of RRP22. Primers used for the GAPDH control were 5′-TAAGGTCTGGATCAACGGATTTTG-3′ and 5′-CATGATGGCCATGAGGTCCACC-3′. PCR products were visualized on 2% agarose gel with added ethidium bromide.

**GTP binding assays.** 293-T cells were transfected with RRP22 constructs and labeled with \( ^{32}P \)orthophosphate overnight. The cells were lysed and the RRP22 immunoprecipitated. The guanine nucleotides were eluted and assayed by thin-layer chromatography on TLC. GTP binding experiments of RRP22 were assayed by loading protein with \( ^{32}P \)GTP as described in ref. (17), releasing bound nucleotide with 20 mmol/L EDTA, and measuring with a scintillation counter.

**Results**

**Sequence comparison of RRP22.** Rig is a Ras-related protein with tumor suppressor properties that is expressed exclusively in brain and heart tissues (4). Using the Rig sequence as a probe, we screened the EST database for similar proteins. We identified RRP22 as sharing 31% identity to Rig and 25% to Ras. This gene has previously been localized to 22q12 (11). Interestingly, this region of the genome exhibits frequent loss of heterozygosity in human tumors, implying that this region of the genome may include tumor suppressor sequences. Sequences were aligned using clustal W (Fig. 1). The RRP22 sequence is notable for several features. Residue 71 (equivalent to Ras 61) is a proline.4 In Ras, changing this residue to proline causes a modest activation of the protein (18). This suggests that RRP22 will be one of the small GTPases that are defective for GTPase activity, like Noe2 and Rheb (19, 20). There is also an insert in the second guanine nucleotide binding site, which may affect the GTPase activity. The RRP22 effector domain (residues 33–42) seems to be unique among Ras-related proteins. Although it shares some homology with Ras and Rig (3 of 8 residues), it also includes an aspartate amino acid insert in the center. Thus, RRP22 is likely to have a unique set of effector proteins. Finally, the COOH-terminal CAAX sequence of RRP22 ends in methionine, suggesting that like Ras and Rig, this protein may be farnesylated (21). Preparation of a dendogram (data not shown) suggested that RRP22 is not simply another member of the Rig/Noe2 family but may form part of an entirely new subgroup.

**RRP22 is farnesylated.** The COOH-terminal CAAX sequence (22) of RRP22 suggests that it will be posttranslationally modified by farnesyl, like Rig and Ras. GST fusion proteins were prepared for RRP22, Rap1a, which is exclusively geranylgeranylated, and H-Ras, which is exclusively farnesylated (23). Equal amounts of the proteins were loaded in a rabbit reticulocyte assay for tritiated lipid incorporation and analyzed by SDS-PAGE followed by autoradiography. Figure 2 shows that all the protein samples labeled equally in the presence of the tritiated isoprenoid precursor mevalonate. H-Ras and RRP22 labeled with farnesyl, but Rap1a did not. Rap1a labeled with geranyl geranyl, but H-Ras and RRP22 did not. Thus, RRP22 is exclusively farnesylated.

**RRP22 is localized to the nucleus/nucleolus.** Ras proteins are classically associated primarily with the plasma membrane, although it is now realized that they may also associate with various other intracellular membranes (24). The localization and functioning of Ras is dependent upon posttranslational modification by farnesyl. It is possible to produce a mutant of Ras that is defective for farnesylation by converting the farnesyl receiving cysteine at the COOH terminus into a serine (6). To investigate the importance of farnesylation for RRP22 function, we manufactured a similar mutant (CAAX-) of RRP22. We then fused both wild-type and CAAX-RRP22 to GFP and examined the subcellular localization of the proteins. Whereas GFP is distributed throughout the cell, K-Ras localized most obviously to the plasma membrane (Fig. 3A, i). Both the wild-type and the CAAX- mutant of RRP22, however, localized to the nucleus and in particular the nucleolus (Fig. 3A, ii and iv). To examine the GTP dependence of the nucleolar localization, we manufactured an equivalent of a Ras mutant that is defective for GFP binding (25). Expression of this mutant (T18N) showed that it no longer localized to the nucleolus. Instead it showed a punctate nuclear pattern that appears to be particularly intense around the periphery of the nucleus (Fig. 3A, v).

To confirm that the nuclear localization of RRP22 was not merely due to the presence of the GFP tag, we did subcellular fractionation experiments on 293-T cells transfected with a FLAG-tagged form of RRP22. FLAG-RRP22 also localized to the nuclear fraction (data not shown).

To confirm that the intense spot/spots within the nucleus were indeed the nucleolus, we cotransfected red fluorescent protein (RFP)–tagged RRP22 and GFP-tagged p14 ARF. p14 ARF has been shown to localize primarily to the nucleolus (26). REP-RRP22 and GFP-p14 ARF colocalized (data not shown). A similar result was obtained for the CAAX- mutant of RRP22. Thus, RRP22 localizes to the nucleolus and this is independent of farnesylation.

**The nucleotide status of wild-type and T18N RRP22.** The structure of RRP22 shows a proline at position 71, equivalent to Ras 61. In Ras, a proline at this position leads to weak activation (18). To examine the GTP dependence of the nucleolar localization, we manufactured a mutant of RRP22, however, localized to the nucleus and in particular the nucleolus (Fig. 3A, ii and iv). To examine the GTP dependence of the nucleolar localization, we manufactured the equivalent of a Ras mutant that is defective for GTP binding (25). Expression of this mutant (T18N) showed that it no longer localized to the nucleolus. Instead it showed a punctate nuclear pattern that appears to be particularly intense around the periphery of the nucleus (Fig. 3A, v).

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4 The RRP22 sequence is deposited as NP_006468.
with GTP/GDP via TLC and autoradiography. Figure 3B, i shows that wild-type RRP22 exhibits a higher degree of association with GTP (≈50%) than does wild-type Ras in cells. A representative example of the TLC assay is shown Fig. 3B, ii. Thus, relative to wild-type Ras, the wild-type RRP22 protein is partially activated.

Examination of the T18N mutant of RRP2 in this assay proved difficult. This was mainly because it gave lower levels of expression than the wild-type protein. To determine the guanine nucleotide–binding characteristics of the T18N mutant, we made GST fusion proteins of the wild-type and T18N mutant and labeled equal quantities of protein in vitro with [α-32P]GTP or [14C]GDP. We then washed the fusion proteins, then eluted bound nucleotide and measured it in a scintillation counter. The T18N mutant of RRP22 seemed to be unable to bind GTP or GDP (Fig. 3B, iii and iv). A similar nucleotide-free state has previously been found in the equivalent dominant negative of RhoA (19N; ref. 27).

RRP22 inhibits growth in a prenylation-dependent manner. To determine if RRP22 acts like Ras or Rig with respect to their effects on cellular growth, we cloned RRP22 into the selectable expression vector pZIP-NeoHABE. We then transfected the plasmids into NIH 3T3 cells and selected in G418. After 2 weeks, live colonies were fixed and stained with crystal violet. Figure 4A shows that RRP22 is a potent inhibitor of cellular proliferation because few colonies survived the experiment with the wild-type protein compared with those transfected with vector. However, colonies did arise in the CAAX/C0 mutant that is defective for farnesylation. Thus, farnesylation is essential for the proliferation-inhibiting activity of RRP22. To determine if the growth-inhibitory properties required a specific type of prenylation, we manufactured an RRP22 mutant with a Rap1a COOH-terminus CAAX sequence that is processed by geranyl geranyl, not farnesyl (RRP22-C20). This mutant retained growth-inhibitory properties and showed localization indistinguishable from the wild-type protein (data not shown). Thus, the activity of RRP22 depends upon isoprenylation, but not specifically farnesylation.

Figure 1. Alignment of RRP22. Sequences were aligned with clustalW. Conserved GTP-binding domains are shown in bold. The predicted effector domain is shown in box 1. Potential activating mutation is shown in box 2 and the CAAX sequence is in box 3.

Figure 2. RRP22 is modified by farnesylation. Two micrograms of RRP22, Rap, and H-Ras 12 V GST fusion proteins were purified from bacteria using glutathione sepharose beads. The protein was then labeled with either 5 μCi [3H]mevalonate (Mev), [3H]farnesyl (FPP), or [3H]geranyl geranyl (GGPP) isoprenoid lipids in a rabbit reticulocyte assay. Proteins were resolved using SDS-PAGE and visualized by autoradiography. Mevalonate labels all isoprenylation-competent protein. Geranyl geranyl labels only Rap1a. Farnesyl labels only Ras and RRP22.
To determine the GTP dependence of the growth-inhibitory properties of RRP22 we did a similar experiment with the T18N mutant. The T18N mutant also inhibited cell survival (Fig. 4A).

**RRP22 promotes cell cycle arrest and caspase-independent cell death.** The effect of RRP22 on cell growth and survival was examined further after overexpression of the protein in a transient transfection system. 293-T cells were transfected with RRP22 and the cells examined for perturbations in the cell cycle by fluorescence-activated cell-sorting (FACS) analysis. Cells showed, on average, an \( \pm \)25% increase in the G2-M phase population, suggesting that they were undergoing cell cycle arrest (Fig. 4B, i). We also did similar experiments wherein the cells were stained with the vital dye trypan blue. RRP22 expression resulted in a significant increase in cells staining blue, showing that wild-type RRP22 could also induce cell death (Fig. 4B, ii). The average of two experiments scored in duplicate is shown in Fig. 4B, iii. The CAAX \(^*\) mutant of RRP22 was severely impaired for its ability to kill the cells, despite being expressed at similar levels to the wild-type form.

To determine if RRP22-induced cell death was apoptotic, we did pSensor assays (14) for caspase activation in live cells transfected with RFP-tagged RRP22. The pSensor indicator protein did not localize to the nucleus, demonstrating that RRP22 is not activating caspases (Fig. 5A). To further analyze the mechanism of cell death, we did TUNEL assays on T-98G human glioblastoma cells transfected with RRP22. We were unable to detect any indication of apoptosis induction by RRP22 by TUNEL assay (Fig. 5B).

**Reintroduction of RRP22 into an RRP22-negative neural tumor cell line impairs the transformed phenotype.** RRP22 mRNA is expressed primarily in the brain (11). Consequently, we examined a variety of neural tumor cell lines to determine if RRP22 was down regulated. Figure 6A shows that RRP22 mRNA is indeed down-regulated in most of the cells when examined by Northern blot. The only positive cell line was the C6 glial cell line. To examine the effect of RRP22 on the tumorigenic phenotype, we reintroduced RRP22 expression in the RRP22-negative glioblastoma cell line,
T-98G. T-98G is a glioblastoma cell line that we have previously found grows well in soft agar. T-98G cells were cotransfected with GFP-RRP22 and a puromycin-selectable vector, pBabe. After selection, pooled populations were examined for their ability to grow in soft agar. The RRP22 cotransfected cells exhibited an ~30% decrease in the ability to form colonies (Fig. 6B, i). Moreover, many of the colonies that did arise were appreciably smaller than those observed in the vector-transfected cells (Fig. 6B, ii). FACs analysis has shown that RRP22 can promote cell cycle arrest (Fig. 4) as well as cell death. Therefore, the reduced growth in soft agar may be due to a combination of growth arrest and death.

**RRP22 is down-regulated by promoter methylation in neural tumor cell lines.** In Fig. 6 we showed that RRP22 expression seems to be frequently down-regulated in tumor cell lines. The only cell line that gave a signal comparable to that of normal brain was the C6 glioma cell line. As this result confirms that RRP22 is expressed in glial cells, we proceeded to analyze the expression and promoter methylation status of the RRP22 in a series of glioma cell lines using a CoBRA-based approach. Here, unmethylated cytosine residues are converted to thymine in the tumor DNA sample. This conversion may be detected by restriction analysis of the PCR DNA (data not shown) or more directly by sequencing of the PCR product of the promoter (Fig. 7A). The results are summarized in Fig. 7B. Most of the tumor cell lines showed extensive promoter methylation. None of the normal tissue samples exhibited promoter methylation.

As a further confirmation that promoter methylation is responsible for the down-regulation of RRP22, we treated some of the tumor cell lines with a demethylating agent, AzaC, and examined the cells by RT-PCR to determine if the gene could be reactivated. All three lines tested showed that in the absence of AzaC, RRP22 expression was undetectable. However, expression of RRP22 was readily detected after treatment with AzaC (Fig. 7C).

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**Figure 4.** A, RRP22 expression inhibits cell growth in a CAAX-dependent manner. NIH 3T3 cells were transfected with 450 ng of RRP22 wild-type or CAAXmut, T18N, or C20 mutants in pZip-NeoHABE. Rig (4) was used as a positive control. Cells were selected in 500 μg/mL of G418 for 2 weeks before fixing and staining with crystal violet. B, RRP22 induces cell cycle arrest and caspase-independent cell death. i, 293-T cells were transfected with GFP-RRP22 and examined by FACs analysis. Representative assay with the average values of three separate experiments in right-hand corner. SE was <20%. ii, 293-T cells were transfected with pcDNAF RRP22 wild-type and CAAXmutant. The cells were stained with trypan blue after 48 hours. Dead cells appear as dark blue dots that may be scored. Bottom, expression levels of RRP22 wild-type and CAAXmutants in the experiment. iii, quantification of the cell death; average of two separate experiments.
Discussion

The discovery of the Noey2/Rig subfamily of Ras-related proteins showed that Ras-like proteins could serve as tumor suppressors as well as oncogenes (3, 4). Noey2 and Rig are frequently down-regulated during tumor development, suggesting that they play an important role in human disease, although their mechanism of action remains largely unknown. With the identification of RRP22 we add a further member to this functional group.

Overexpression of RRP22 causes growth inhibition and cell death in a variety of cell lines. The mechanism behind the cell death remains unknown but, like Noey2 (28), RRP22 does not seem to promote caspase activation. We were also unable to detect apoptosis in T-98G cells transfected with RRP22 by TUNEL assay.

Like Rig and Noey2, RRP22 has a potentially natural “activating mutation” in its primary structure. Our analysis of the GTP/GDP ratio of the wild-type RRP22 confirmed that it is partially activated by showing that the protein is ~50% associated with GTP. Several other members of the Ras superfamily also show this feature, for example, Rheb and Noey2 (19, 20). One explanation for this finding may be that these proteins do not function as GTPases at all and lack the normal guanine nucleotide exchange factor and GTPase-activating protein (GAP) regulators. However, Rheb has now been shown to be a target for the Tsc2 GAP activity (29). Moreover, mutagenic analysis of Rheb strongly suggests that it is regulated by exchange factors (17). Thus, despite the intrinsically activated state of these proteins, it is still likely that they may be regulated in the standard manner by as yet unknown GAPs and guanine nucleotide exchange factors. This possibility is supported by our observation that the T18N mutant of RRP22 is biochemically analogous to the dominant-negative RhoA mutant 19N (27). This mutant is defective for nucleotide binding and binds Rho exchange factors with high affinity. The growth-inhibitory properties of RRP22 T18N suggest that proteins that share exchange factors with RRP22 may be essential for cell survival.

The key region of a Ras-like protein for dictating its biological function is the small effector domain between Ras residues 32 to 40 (30). The composition of this domain largely mandates which downstream targets the Ras-like protein will bind. The Rig and Ras effector domains are sufficiently similar to allow both to bind the Ras effector Raf-1. However, The RRP22 effector domain is unique, sharing only three of eight amino acids with Rig or Ras. Moreover, it has a single amino acid “insert” in the center. This property is unique and suggests that it may be able to bind a quite different set of effector proteins to those of Ras, Rig, and Noey2. Indeed, RRP22 does not bind Raf-1 (data not shown).

The final obvious conserved domain in RRP22 is the last four amino acids, which make up a CAAX box. Typically, CAAX boxes ending in methionine have been found to be compatible with modification with the farnesyl lipid, like Ras (21). Indeed, RRP22 seemed to be exclusively modified by farnesyl in vitro. One of the reasons that we were interested in studying RRP22 was to determine if it might play a role in the cellular response to farnesyl transferase inhibitors. Like Ras, the processing of RRP22 is critical to its function, as a CAAX-defective mutant lost the ability to inhibit growth despite being expressed at similar levels to the wild-type protein. Thus, exposure to farnesyl transferase inhibitor drugs may actually serve to inactivate the tumor suppressor properties of RRP22. This observation may serve as a cautionary note to any attempt at long-term use of farnesyl transferase inhibitor compounds.

K-Ras is normally farnesylated but retains the ability to transform cells as long as it is processed by farnesyl or geranyl geranyl (31). Likewise, we found that RRP22 needs to be isoprenylated to inhibit growth but that a geranylgeranylated mutant of RRP22 was as effective as the wild-type.

Ras proteins were originally described as localizing to the plasma membrane, although it is now apparent that they also reside on other membrane surfaces (24). However, examination of RRP22 both by fluorescent microscopy in live cells and by subcellular fractionation clearly shows that this protein localizes to the nucleus and in particular the nucleolus. This intrigued us because no other prenylated Ras-like protein has been reported in the nucleolus. Analysis of a GTP-defective mutant of RRP22

![Figure 5](image-url)

**Figure 5.** A, RRP22-mediated cell death is caspase independent. phcRed-RRP22 was cotransfected into 293-T cells with pCaspase3-Sensor. RRP22 positive cells were examined for the relocation of the Green pCaspase3-Sensor reporter protein to the nucleus. No such relocation was observed although such relocation was clearly visible in cells expressing the proapoptotic RASSF2 positive control. B, RRP22-mediated cell death does not appear to be apoptotic. TUNEL assays were done on T-98G cells transfected with GFP-RRP22 using a Deadend kit (Promega). Green cells were scored for positive TUNEL staining. Values were normalized to the Fas positive control.
(T18N) suggested that when not bound to GTP, RRP22 localizes outside of the nucleolus. This implies that RRP22 cycles in and out of the nucleolus in a GTP-dependent manner. This observation would be compatible with a role in transporting material in and out of the nucleolus. In this case, RRP22 would be functionally analogous to the Ras-related Rab family of proteins that regulate intracellular transport. Interestingly, a dominant-negative form of Rab, equivalent to the T18N mutant of RRP22, blocks transport and inhibits cell growth (32).

The nucleolar localization of RRP22 was confirmed by demonstrating that RRP22 colocalizes with p14 ARF, a known nucleolar protein. p14 ARF is one of the few proteins that have previously been characterized as a nucleolar tumor suppressor. The mechanism by which p14 ARF functions remains under debate (33, 34), but it has recently been shown to play a key role in regulating ribosome biogenesis in the nucleolus (35). It is now being realized that aberrations in the regulation of ribosome production may be a key aspect of tumor development (36). Consequently, we investigated the possibility that RRP22 was functioning via p14 ARF. However, we have been unable to show stable association of RRP22 with p14 ARF, and the T18N mutant of RRP22 does not alter the nucleolar localization of p14 ARF (data not shown). Moreover, initial experiments examining the effects of RRP22 on an rDNA luciferase reporter failed to show any inhibition of nucleolar RNA polymerase I (data not shown). Thus, the precise function of RRP22 remains under investigation.

The nucleolar localization of RRP22 is not due to isoprenylation inasmuch as the CAAX\textsuperscript{\textendash} mutant is also nucleolar. If the CAAX\textsuperscript{\textendash} mutant does not mislocalize, why is it biologically inactive? One possible explanation of this effect may be extrapolated from the interaction of Ras with its effector, Raf-1. Farnesylated Ras shows a considerably higher binding affinity for Raf-1 than unprocessed Ras (7). Perhaps the CAAX\textsuperscript{\textendash} mutant of RRP22 is likewise less effective at binding its targets and consequently acts like a null mutant.

The expression pattern of RRP22 is exclusive to neural tissue (11). Microarray analysis of primary neural tumors has shown that loss of RRP22 expression is one of the markers that correlates with poor prognosis (37) and we have now found that most neural tumor cell lines lose expression of RRP22. Because the mechanism by which the related Noey2 protein is inactivated in human tumors involves promoter methylation (3), we sought to determine if the same was true for RRP22. Our analysis of a series of human neural tumor cell lines shows that this is indeed the case. This type of inactivation is commonly observed in a wide range of tumor suppressors in human cancer (38).

Finally, to confirm that RRP22 could serve as a tumor suppressor, we transfected an RRP22 expression construct into...
an RRP22-negative human neural tumor cell line. The ability of the cell line to grow in soft agar was impaired.

In summary, we have identified a novel, farnesylated member of the Ras superfamily that exhibits the properties of a potential neural-specific tumor suppressor and is implicated in the regulation of nucleolar transport processes.

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**References**


**Figure 7.** Direct sequencing shows the RRP22 CpG island predicted promoter region is heavily methylated in glioma tumor cell lines. A, unmodified sequence showing the CpG island region analyzed. Numbered are the CpG dinucleotides within the region analyzed and the position of the first and last nucleotides relative to the transcription start site of the RRP22 gene (NM_006477). Light gray indicates the predicted promoter region at −262 > +333 bp (PromoterInspector, available at www.genomatix.de). Dark gray, locations of the primers used to amplify the region using PCR. The CpG island was identified using CpG plot (www.ebi.ac.uk). It is located at −490 > +1993 bp relative to the transcription start site of RRP22 (NM_006477) and has an ObsExp of 1.07 and a CG percentage of 62.16. Dashed box, ATG translation start site. B, direct sequencing shows methylation is widespread across the entire region analyzed. ■, completely methylated; □, partially methylated; □, unmethylated. N1, N2, and N3, DNA from normal human adult brain tissue. SAM, artificially methylated DNA, serving as a positive control. C, hypermethylation of the RRP22 CpG island correlates with loss of RRP22 expression and treatment with a demethylating agent reactivates gene expression. RT-PCR shows reactivation of RRP22 expression after AzaC (+) in three methylated glioma tumor cell lines. GAPDH expression was used as a control for equal loading and RNA integrity.
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