The Human Tumor Suppressor ARF Interacts with Spinophilin/Neurabin II, a Type 1 Protein-phosphatase-binding Protein*

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The INK4a gene, one of the most often disrupted loci in human cancer, encodes two unrelated proteins, p16INK4a and p14ARF (ARF) both capable of inducing cell cycle arrest. Although it has been clearly demonstrated that ARF inhibits cell cycle via p53 stabilization, very little is known about the involvement of ARF in other cell cycle regulatory pathways, as well as on the mechanisms responsible for activating ARF following oncoproliferative stimuli. In search of factors that might associate with ARF to control its activity or its specificity, we performed a yeast two-hybrid screen. We report here that the human homologue of spinophilin/neurabin II, a regulatory subunit of protein phosphatase 1 catalytic subunit specifically interacts with ARF, both in yeast and in mammalian cells. We also show that ectopic expression of spinophilin/neurabin II inhibits the formation of G418-resistant colonies when transfected into human and mouse cell lines, regardless of p53 and ARF status. Moreover, spinophilin/ARF coexpression in Saos-2 cells, where ARF ectopic expression is ineffective, somehow results in a synergic effect. These data demonstrate a role for spinophilin in cell growth and suggest that ARF and spinophilin could act in partially overlapping pathways.

The INK4a gene, one of the most frequently disrupted loci in human cancer (1–3) gives rise to two distinct transcripts from different promoters (4). Each transcript has a specific 5′-exon, E1α or E1β, and two common exons E2 and E3. The E1α-containing transcript encodes p16INK4a, which acts as an inhibitor of cyclin-dependent kinases 4 or 6 and prevents the phosphorylation of pRb (5), thereby maintaining an active pRb and blocking the exit from the G1 phase. The E1β-containing transcript encodes ARF (a 14-kDa polypeptide in humans, 19 kDa in mouse). Mouse and human ARF proteins are 45% identical through their exon 1β segments and 50% identical overall (6). ARF inhibits cell growth by interacting with MDM2 (7–10), which is a multifunctional protein that negatively regulates p53 in several ways. First, its binding interferes with p53's ability to transactivate target genes (11). Second, MDM2 has an intrinsic ubiquitin ligase activity that most likely contributes to p53 degradation (12). At least in vitro, ARF can interfere with this reaction (13), but whether this is central to ARF actions in vivo is unknown. Third, MDM2 relocates p53 from the cell nucleus to the cytoplasm where it undergoes proteosomal degradation (14). Both mouse and human ARF are nucleolar proteins. When coexpressed with MDM2 or induced by conditional Myc expression, ARF relocates MDM2 to the nucleolus, preventing MDM2 shuttling and stabilizing p53 in the nucleolasm, thereby prompting cell cycle arrest (15–17). In principle, ARF may antagonize any or all of the MDM2 functions. On the other hand, the antagonism of MDM2 by ARF could potentially affect functions of proteins other than p53, such as E2F-1 (18), pRb (19), p300/CBP (20), and other p53 family members (21).2

The induction of ARF by oncoproteins such as Myc, E1A, Ras, and v-Abl (22–25) highlights its role in sensing hyperproliferative signals in incipient cancer cells, and because ARF is also induced by E2F (26) it biochemically connects the pRb and p53 pathways.

Furthermore, although it has been clearly demonstrated that ARF inhibits cell cycle via p53 stabilization, very little is known about the involvement of ARF in other cell cycle regulatory pathways (27, 28), as well as on the mechanisms responsible of ARF activation by oncoproliferative stimuli.

To identify and isolate proteins important for conferring functional specificity to ARF, we employed a yeast two-hybrid screen. In this paper we report the isolation of the human homologue of the rat spinophilin (also known as neurabin II), a regulatory subunit of protein phosphatase 1 catalytic subunit

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™ and EBI Data Bank with accession number(s) AJ401189 and HSA401189.

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1 The abbreviations used are: ARF, protein p14ARF; PP1, protein phosphatase 1, PP1c, protein phosphatase 1 catalytic subunit; bp, base pairs; PCR, polymerase chain reaction; aa, amino acid(s); PBS, phosphate-buffered saline; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; CFE, colony formation efficiency; kb, kilobase(s); ABD, F-actin-binding domain; GFP, green fluorescence protein; MBP, maltose-binding protein; PDZ, PSD-95/discs large/ZO-1 domain.

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(PP1c) (29–31), and demonstrate the specific interaction between ARF and spinophilin. PP1 is one of the major serine/threonine-specific protein phosphatases in eukaryotic cells (32) involved in controlling diverse cellular functions, including the exit from mitosis and splicing of mRNAs (33, 34). PP1 has been involved in controlling diverse cellular functions, including the dephosphorylation of specific residues of p53 (35).

We also show that spinophilin is able to inhibit the formation of G418-resistant colonies when transfected into human and mouse cell lines regardless of p53, pRb, and ARF status. These data suggest a role for spinophilin in cell growth.

**EXPERIMENTAL PROCEDURES**

**Plasmids**

**Yeast Two-hybrid Screening**—The EcoRI/Sall fragment encoding the entire ARF (132 amino acids) was excised by p19 plasmid (3) and cloned into the yeast two-hybrid bait vector pBTM116 (34) to generate pBTM-ARF. The entire ARF (132 amino acids) was excised by p19 plasmid (3) and cloned in pcDNA-HisA (pcDNA-ARF). To obtain the N-terminal region of spinophilin (SpinoC) was cloned in pRcCMV (3) cut by EcoRI/BamHI. The fragment encoding the amino acids 552–813 of spinophilin, the HindIII/BglII fragment (encoding amino acids 1–813), excised from pCMV-Spinophilin was cloned in pGFP-C3 cut with EcoRI/BamHI, purified, and religated.

**In Vitro Protein-Protein Interaction**—The 636-bp fragment encoding the C-terminal region of spinophilin (SpinoC) was cloned in pGEX-4T1 (Amerisham Pharmacia Biotech) to obtain pGEX-SpinophC plasmid. EcoRI/Sall fragment excised from p19 plasmid was cloned in pMAL-c2 (New England Biolabs, Hitchin, Hertfordshire, Great Britain) to obtain the plasmid pMAL-ARF. The EcoRI/HindIII fragment encoding the full PP1c coding sequence was excised from pYES-PP1c plasmid (36) and cloned in pBAD-HisA (Invitrogen) plasmid (pBAD-PP1c).

**Yeast Two-hybrid Screen**

pBTM-ARF vector was used to screen a human brain cDNA library cloned into the pGAD10 vector (CLONTECH, Palo Alto, CA). The yeast strain L40 (34) was sequentially transformed with the pBTM-ARF vector and the library. An estimated 10^7 transformants were screened. Yeasts containing interacting proteins were identified by growth on selective media lacking leucine, tryptophan, and histidine and encoding amino acid activity. Isolated plasmids were rest-ransformed into L40 with the negative control plasmid pBTM-galactin (a gift of L. Chiarotti) and with pBTM-ARF and tested again for growing on the selection media. Those that were negative for interaction with galactin were sequenced and DNA sequences were used to search the non-redundant GenBank® data base using the BLAST search algorithm (37) available from the National Institutes of Health on the Web.

**Anti-ARF Antibody Preparation and Purification**

The MBP-ARF fusion protein was obtained by expression in TG1 Escherichia coli strain transformed with pMAL-ARF plasmid and purified on amylose-agarose (Amerisham Pharmacia Biotech) following the procedure suggested by the manufacturer. The protein was further purified by gel-filtration on S-300 (Amersham Pharmacia Biotech). Anti-ARF polyclonal antibodies were purified by papain digestion and isolated plasmids were re-transformed into L40 with the negative control plasmid pBTM-galactin (a gift of L. Chiarotti) and with pBTM-ARF and tested again for growing on the selection media. Those that were negative for interaction with galactin were sequenced and DNA sequences were used to search the non-redundant GenBank® data base using the BLAST search algorithm (37) available from the National Institutes of Health on the Web.

**AF, Spinophilin, and PP1c in Vitro Interaction**

The GST:SpinophC fusion protein was obtained by expression in TG1 E. coli strain transformed with pGEX-SpinophC plasmid and purified by affinity chromatography on glutathione Sepharose 4B (Amerisham Pharmacia Biotech) following gel filtration, in PBS buffer, on Superdex 200 (Amerisham Pharmacia Biotech). MBP and MBP-ARF were expressed in E. coli and purified by affinity chromatography on amylose-agarose (New England Biolabs, Hitchin, Hertfordshire, Great Britain) followed by gel filtration, in PBS buffer, on S-300 (Amerisham Pharmacia Biotech). Approximately 3 μg of purified MBP or MBP-ARF were mixed with the same amount of GST or GST:SpinophC in TNN buffer (50 μl, 50 μM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 10% NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% bovine serum albumin) and (25 μl of gel volume) amylose-agarose (New England Biolabs, Hitchin, Hertfordshire, Great Britain) was added (20 min at 4 °C). The beads were collected by centrifugation and washed three times with TNN buffer containing 1 μl urea and twice with TNN buffer. Samples were loaded onto 10% SDS-PAGE, blotted on nitrocellulose paper (Schleicher & Schuell), and probed with...
anti-GST antibody (1:2000; Sigma Chemical Co., St. Louis, MO) followed by horseradish peroxidase (HRP) anti-g GST immunoglobulin G (1:1000; Sigma). Immuno reactive bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, UK). Hi6::Xpress::PP1c was obtained by expression in TOP10 E. coli strain (Invitrogen) transformed with pGAD-PP1c. Induction and purification on nickel chelating resin (Invitrogen) was performed as suggested by the manufacturer. PP1c and MBP::ARF coprecipitation experiment was performed essentially as described above.

**Far Western**

Two aliquots of purified MBP::ARF were separated on 10% SDS-PAGE, transferred to nitrocellulose, and blocked in PBS containing 3% dried skimmed milk (blocking buffer) for 1 h at 37 °C. Filters were separately incubated with GST::SpinoC and GST (50 μg/ml, 18 h, 4 °C), followed by anti-GST polyclonal antibody (1:2000, 2 h, 37 °C) and by HRP-anti-g GST immunoglobulin G (1:1000, 2 h, 37 °C). Immunoreactive bands were visualized by enhanced chemiluminescence.

**Cell Culture, Transfection, Coimmunoprecipitation, and Cell Imaging**

All cells were cultured in a 37 °C incubator with 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 2% glucose, penicillin/streptomycin 100 units/ml each and 10% fetal bovine serum except for NIH3T3 cultured in the same medium supplemented with 10% calf serum.

For coimmunoprecipitation assay, COS-7 cells were seeded into a 6-well plate (2.5 x 10⁵ cells per well) and transfected using the standard calcium phosphate method (39). Forty-eight hours after transfection the cells were replated in a 100-mm dish and selected using the standard calcium phosphate method (39). Forty-eight hours later 10% methanol/10% acetic acid and stained with crystal violet 15 days post-transfection. The colonies were counted. Each experiment was repeated at least four times.

**RESULTS**

**Identification of Spinophilin as an ARF-binding Protein**—In an attempt to identify human proteins that interact with ARF, we have used the yeast two-hybrid system (38). The entire ARF cDNA was fused to the GAL4 DNA-binding domain (BD). As detailed under “Experimental Procedures” this construct was used as bait to screen a human brain cDNA library from a screen of ~10⁷ yeast transformants, 30 colonies were scored as positive for reporter gene activity (His⁺/LacZ⁺). Among 19 clones scored positive in secondary screening assays, 18 encoded a 212-amino acid polypeptide (pGAD-SpinoC), which was identified in a data base search as the human homologue of the C-terminal portion of rat spinophilin/neurabin II (31, 37). The last clone (pGAD-B2) differed in length only by 177 bp at the 5’-end (Fig. 1A). The full-length cDNA (EMBL accession numbers AJ401189 and HSA401189), reconstructed as detailed under “Experimental Procedures,” encodes a 813-amino acid long polypeptide showing 95% amino acids identity (Fig. 1B) with the rat spinophilin/neurabin II (GenBank® accession number AAB72005). Performing a BLAST search on the GenBank® data base we have also identified a cosmid derived from the human chromosome 17 (GenBank® accession number AC002401) containing the gene sequence of human spinophilin, which consists of 12 exons spanning over a region of 15 kb. The so-called spinophilin was first identified in rat as a cellular partner of type 1 protein phosphatase (PP1), which is one of the main eukaryotic serine/threonine protein phosphatases involved in the control of cell cycle progression. Rat spinophilin is characterized by an N-terminal domain (aa 1–295), in which are present various putative Src homology 3 binding motifs, and by a C-terminal domain being a protein phosphatase 1 (PP1c)-negative regulator (295–817). Spinophilin sequence contains various protein-protein interaction signals spread throughout the whole sequence (Fig. 1A); an F-actin binding domain (ABD), a PP1c binding site (R/K)(V/I), a PDZ domain, and a myosin-like left handed α-helix. Both pGAD-SpinoC and pGAD-B2 encode the predicted coiled-coil region (Fig. 1A) observed in rat spinophilin (31) and pGAD-B2 encodes also a portion of the PDZ domain (Fig. 1A). Both clones showed interaction with ARF in our yeast two-hybrid system, leading us to draw the conclusion that the coiled-coil region of spinophilin is the binding domain for ARF.

**In Vitro Binding Assays and Coimmunoprecipitation**—To confirm the ARF/spinophilin interaction we performed coprecipitation and coimmunoprecipitation experiments. We expressed ARF as MBP fusion (MBP::ARF) using the pMal-c2 system and the 212-amino acid C-terminal region of spinophilin (SpinoC) as glutathion S-transferase fusion (GST::SpinoC) using the pGEX system. Purified MBP::ARF or MBP were incubated with either purified GST::SpinoC or GST, and amylose-agarose beads were added. After extensive washing the bound proteins were separated on SDS-PAGE, blotted, and analyzed using anti-GST polyclonal antibodies. As shown in Fig. 2A, GST::SpinoC coprecipitated with MBP::ARF (lane 1), but not with GST (lane 2), and MBP did not bind to GST::SpinoC (lane 3). The GST::SpinoC interaction with MBP::ARF was further confirmed by far-Western blotting. Two aliquots of purified MBP::ARF were analyzed on SDS-PAGE and blotted onto nitrocellulose. Ponceau S staining (data not shown) confirmed the presence of an equal amount of proteins on the blots. The blots were probed, respectively, with an equal amount of GST::SpinoC and GST followed by incubation with anti-GST antibodies. As shown in Fig. 2B the anti-GST antibody only binds to the filter probed with GST::SpinoC (lane 1).

Although the in vitro coprecipitation experiments clearly indicate that the C-terminal region of spinophilin is able to bind ARF we also confirmed the interaction in intact cells. COS-7 cells, a mammalian cell line known for its robust expression of recombinant proteins, were transfected with mammalian expression plasmids encoding Xpress-tagged spino philin, or Xpress-tagged human MDM2, and/or ARF. The cellular lysates were immunoprecipitated with anti-ARF antibodies. The immunoprecipitates were blotted and probed with anti-Xpress antibodies. Coimmunoprecipitation of spinophilin (Fig.
2C, lane 3) as well as human MDM2 (Fig. 2C, lane 4), which has been reported to interact with ARF (7), occurred only when each of these proteins was coexpressed with ARF, because the complexes were not found when either protein alone was introduced into the cells (Fig. 2C, compare, lanes 3 and 4 to lanes 5 and 6). Furthermore, in a similar experiment in which plasmids encoding ARF and/or, respectively, the N-terminal region (SpinoN) or the C-terminal region of spinophilin (SpinoC) (Fig. 2D) were transiently transfected in COS-7 cells, only the C-terminal region of spinophilin coimmunoprecipitated with ARF (Fig. 2D, lane 6) confirming that the ARF-binding domain is localized in the C-terminal region of spinophilin.

**ARF-Spinophilin Minimal Interaction Requirements**—To identify the minimum region of ARF essential for the interaction with spinophilin, two deletion mutants of ARF were assayed for the interaction with spinophilin in yeast (Fig. 3). Our yeast two-hybrid analysis showed that region 1–65 of ARF, corresponding to the region encoded by exon 1β (1–65 aa, Fig. 3A, lane b), is required for the interaction with the C-terminal region of spinophilin. The deletion of either the last 27 amino acids (Fig. 3A, lane c) or of the amino acids 1–38 of exon 1β encoded region (Fig. 3A, lane d) impairs the interaction with spinophilin, suggesting that the entire region encompassing amino acids 1–65 is essential for the binding to spinophilin.
To identify the minimum region of the C-terminal part of spinophilin required for interaction with ARF we have created various deletion mutants. The mutants design was based on the prediction, using the COILS program (38), of the coiled-coil region (673–813) already observed in the rat spinophilin (31).

The C-terminal region of spinophilin (605–813) encoded by the pGAD10-SpinoC is divided in four regions: non-helix (605–673, black rods), helix A (674–726, white rectangles), helix B (727–788, black rectangles), and helix C (789–813, gray rectangles).

We have sequentially deleted the helices from C to A and analyzed the ability of the mutants to bind to ARF. Our yeast two-hybrid data showed that helices C (Fig. 3A, lane e) and B (Fig. 3A, lane f) are not required for the interaction with ARF whereas the C-terminal mutant, which also lacks the helix A (Fig. 3A, lane g), failed to interact with ARF. To obtain an independent evaluation of the protein-protein interaction suggested by the two hybrid results, we examined the ability of the described deleted peptides to coimmunoprecipitate with ARF in cell extracts. COS-7 cells were transfected with mammalian expression plasmids encoding Xpress-tagged spinophilin or Xpress-tagged human MDM2, and/or ARF. Cellular extracts were incubated with anti-ARF antibody and precipitated with protein A-Sepharose beads. Samples were analyzed by immunoblotting with anti-Xpress antibody. Anti-ARF antibodies were unable to immunoprecipitate spinophilin and the N-terminal and C-terminal deletion mutants without the co-expression of ARF (lanes 2–4). Only the C-terminal domain of spinophilin and the full-length protein could be detected after co-immunoprecipitation when ARF was coexpressed (lanes 6 and 7).

Biological Activity of Spinophilin—The interaction between ARF and spinophilin, a regulatory subunit of protein phosphatase 1, raises the question of the biological significance of this interaction. PP1 is one of the major serine/threonine-specific protein phosphatases in eukaryotic cells (32), which has a crucial role in the cell cycle progression (33–35). In principle, a direct interaction could be possible between PP1 and ARF. Careful examination of the ARF sequence revealed the lack of the PP1c pentapeptide motif ([R/K][R/K][V/I]XF) conserved in
all the PP1 regulatory subunits (29). Moreover, coprecipitation experiments were performed using purified MBP::ARF and purified His6::PP1c, and we did not observe any specific interaction between these two proteins (data not shown), although we cannot exclude that a trimeric complex could exist.

Because ectopic expression of ARF results in growth suppression, to assess a possible role of the ARF/spinophilin interaction, we analyzed the effect of spinophilin in growth suppression by colony formation efficiency assay (CFE) using different human and mouse cell lines. Saos-2 cells express ARF and are p53 and pRb null (9), whereas U2OS cells express wild type p53 and pRb and show a near undetectable level of ARF expression, and the NIH3T3 cell line bears a deletion of the INK4a locus. We transfected expression vectors encoding ARF or the entire spinophilin, into all three cell lines. After completion of drug selection, growth suppression was quantified by comparing the relative number of drug-resistant colonies obtained with each construct to that obtained with the empty vector. As already reported (3) ARF strongly inhibits the G-418R colony formation only in the U2OS and NIH3T3 cell lines, which express both p53 and pRb (Fig. 4). Interestingly, spinophilin appears to reduce the number of resistant colonies with an efficiency similar, if not higher, to that exhibited by ARF, in both cell types. A more complex picture derives from the experiments in Saos-2 cells, where ARF ectopic expression is ineffective, with spinophilin reducing the number of G-418R colony up to 50% with respect to the control only when the higher amount of expression vector was used (Fig. 4). These data indicate a role for spinophilin in cell growth that is independent from the status of p53, pRb, and ARF.
lin and ARF, which alone have little or no effect on colony formation, reduces the number of colonies significantly, suggesting a synergy between the two proteins in this cell line (Fig. 4).

Transfection of expression vectors encoding, respectively, the C-terminal region or the N-terminal region of spinophilin resulted in a number of colonies almost similar to that of the control vector, suggesting that the integrity of the protein in all cell lines is required for the biological activity (data not shown). On the other hand, the effect of the transfection of 1 μg of ARF did not vary when either the C-terminal region or the N-terminal region of spinophilin where coexpressed (data not shown).

**Cellular Localization**—Spinophilin/neurabin II is ubiquitously expressed and has been shown to localize in rat at the level of the dendritic spines (31). It has been shown that in neuronal tissues rat spinophilin/neurabin II is involved in the binding of various proteins usually found in the cytoplasm, F-actin (39), TGN38 (40), D2 dopamine receptor (41), and Lin-10 (42). ARF has a preferential localization in the nucleus, but, when overexpressed with MDM2 and p53, it has also been found in “nuclear bodies” within the nucleoplasm (16). Because our results clearly show an interaction in vitro and in intact cells between ARF and spinophilin, we decided to assess the intracellular localization of spinophilin in mammalian cell lines. Consequently, we transfected COS-7 and NIH3T3 cell lines with GFP fusion constructs of either ARF or spinophilin. To verify that addition of the GFP did not alter the biological activity of these proteins, we performed CFE experiments using GFP fusion constructs of either ARF or spinophilin. The results were similar to that obtained with the expression vector lacking the GFP, indicating that addition of the reporter protein did not alter the biological activity of these proteins (data not shown).

Our results show that spinophilin, at least in COS-7 and NIH3T3 cells, is expressed both in the nucleus and in the cytoplasm (Fig. 5C). To define the minimum region of spinophilin needed for the nuclear localization, two deletion mutants of GFP::Spinophilin were constructed and assayed in COS-7 and NIH3T3 cells. Our localization data suggest that the spinophilin region necessary for nuclear localization is the region encompassing amino acids 552–813, because this region is deleted in the GFP::SpinoN mutant (1–473), which is clearly excluded from the nucleoplasm (Fig. 5E). On the other hand, the GFP::SpinoC mutant bearing the region encompassing amino acids 552–813 is localized both in the nucleus and in the cytoplasm (Fig. 5D).

**DISCUSSION**

Biochemical evidence supporting the genetic interaction between ARF and p53 comes from the finding that ARF physically interacts with MDM2 and consequently stabilizes p53, but the molecular pathway by which ARF stabilizes p53 is not clear at present (15–17). Moreover, very little is known about the involvement of ARF in other cell cycle regulatory pathways, as well as on the mechanisms responsible for the activation of ARF by oncoproliferative stimuli. However, the existence of additional ARF-interacting proteins was clear from our preliminary data, and in this paper we have described the identification of a new partner of human ARF, spinophilin. Our studies have shown that an intact ARF N-terminal region (aa 1–65) is necessary for this interaction, because deletion of either the last 27 or the first 1–38 amino acids of this domain impairs the interaction. Although further experiments are necessary for a more refined definition, these results suggest that the ARF

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3 V. Calabrò, G. Mdnsueto, T. Parisi, M. Vivo, R. Calogero, and G. La Mantia, unpublished results.
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restrict the location of kinases and phosphatases but also modifies their catalytic and regulatory properties and allows their activity to be regulated by extracellular signals. Spinophilin belongs to this class of regulators, because it negatively regulates PP1 activity. PP1 is one of the main eukaryotic serine/threonine protein phosphatases involved in the control of cell cycle progression (reviewed in Ref 35), and it has been implicated in the mitotic dephosphorylation of pRb (35), as well as in the dephosphorylation of specific residues of pS3 (35, 46). PP1 is found associated with pRb in the G2 phase and during mitotic exit (47). This temporal association between PP1 and pRb appears to have a functional significance in that it coincides with the reactivation of pRb-mediated growth suppression (48). The observation that PP1d isoform containing the greatest pRb-directed activity is found associated with a 110-kDa interacting protein (49) again underlines the importance of the interacting subunits of phosphatases.

Before PP1 activity was ever implicated in the regulation of pRb, it was known to have a role in the regulation of mitosis and cell division. PP1 mutations in Drosophila (50), yeast (51), and fungi (52) displayed varied mitotic defects and different degrees of lethality. Miotic blocks were observed upon microinjection of PP1-neutralizing antibodies (53) and PP1 inhibitors such as okadaic acid (54, 55). In addition to this, the distribution of PP1 changes with progression of the cell cycle, accumulating at the nucleus to associate with chromatin during G2 and M phase (55). Potential targets for PP1 in the nucleus are the mitotic cyclin B/cdk1 substrates that include histone H1, lamin, microtubule-associated proteins, and perhaps other proteins that have yet to be identified.

Strikingly, spinophilin appeared to be a strong growth suppressor in CFE assays in both human U2OS and mouse NIH3T3 cells that are wild type for both p53 and pRb but do not express ARF proteins. In U2OS cells coexpression of ARF and spinophilin resulted in a stronger effect on inhibition of colony formation, suggesting an additive effect between the two proteins. Surprisingly, in Saos-2 cells, where ARF is unable to suppress colony formation, as already reported (3), spinophilin suppress colony formation, suggesting an additive effect between the two pro-

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