The human tumor suppressor ARF interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein

Running title: Interaction of ARF with neurabin II/spinophilin, a PP1-binding protein

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ABSTRACT

The INK4a gene, one of the most often disrupted loci in human cancer, encodes two unrelated proteins, $p16^{INK4a}$ and $p14^{ARF}$ (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 (PP1c) specifically interacts with ARF, both in yeast and in mammalian cells. We also show that ectopic expression of spinophilin/neurabinII 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.

INTRODUCTION

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 $p16^{INK4a}$, 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 relocalizes 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 relocalizes MDM2 to the nucleolus, preventing MDM2 shuttling and stabilizing p53 in the nucleoplasm, 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) (Calabrò et al. submitted). 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 since 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 catalitic subunit (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 implicated in the mitotic dephosphorylation of pRb (35), as well as in 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/SalI 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.

Exon 1β construct*:* The exon 1β was excised by EcoRI digestion from pN-p19 plasmid (3) and cloned in pBTM116 cut by EcoRI (pBTM-ARF-(1-65)).

ARF deletion mutants: pBTM-ARF-(1-38) was obtained by cutting with EcoRI/NarI pBTM-ARF and cloning the 114 bps fragment in pUC19 cut by EcoRI/AccI (pUC19-ARF-(1-38)). The insert EcoRI/PstI was cloned in pBTM digested with the same enzymes. pBTM-ARF-(39-132) was obtained by cutting with NarI/HindIII pBTM-ARF and cloning the 436 bps fragment in pUC19 cut with AccI/HindIII (pUC19-ARF-(39-132)). The EcoRI/HindIII(filled-in) fragment, excised from pUC19-ARF-(39-132) was inserted in pBTM cut by EcoRI/SalI(filled-in).

Full length spinophilin constructs: BamHI/EcoRI fragment (1100 nts), encoding the C-terminal portion of spinophilin, excised by clone B2 was cloned in pGEM (pGEM-B2). The amplification from a human cDNA brain library (CLONTECH, UK) of the N-terminal region was done using two oligonucleotides ATGSP (5' GCTCCAAGCTTCATGATGAAGACGGAG 3') and RSPBAM (5' ACCAGGAGATCGTTCACTTGGATCCT 3'), designed on the published sequence of the rat spinophilin (GenBank Accession Number AF016252). The 1650 bps amplified fragment was digested with HindIII/BamHI, releasing a HindIII/BamHI fragment (1450 bps containing the spinophilin ATG) and a BamHI/BamHI fragment (200 bps, located downstream the 1450 bps fragment). The 1450 bps fragment was cloned in pGEM-B2 cut by HindIII/BamHI (pGEM-

N-spino-BamHI-C-spino). The 200 bps fragment was cloned in the plasmid PGEM-N-spino-BamHI-C-spino cut by BamHI (pGEM-Spinophilin, 2500 bp). The plasmid pGEM-Spinophilin was used for coupled transcription/translation in vitro using the TNT T7 Quick Coupled Transcription/Translation System (Promega, USA). pGEM-Spinophilin was sequenced and the sequence was submitted to the Nucleotide Sequence Database at EMBL (EMBL Accession Numbers AJ401189, HSA401189).

Spinophilin deletion mutants: All the C-terminal mutants were obtained by inverse PCR using as template pGAD10-SpinoC (encoding aa 605-813) and the common GAD10c primer (5' CGTCTAGATATGAATCGTAGATACTGAAAAACCCCGCAAGTTC 3'). The GAD10c primer was used together with the NH-AB primer (5' CGTCTAGATTACTCCGACTCCTCCAGAACCCGACGCTG 3') to obtain pGAD-nH-AB (encoding aa 605-787); with the NH-A primer (5' CGTCTAGATTAACCCCAGTAGCCTTCCAGTTTCTCCATGCG 3') to obtain pGAD-nH-A (encoding aa 605-728); with the NH primer (5' CGTCTAGATTACAGCTTCTCGGGCTCCATGTCCACAGG 3') to obtain pGAD-nH (encoding aa 605-668). Inverse PCR was performed using the "Long Range PCR Kit" (Boehringer, D) following the condition suggested by the manufacturer. Amplified mutants cut by XbaI were purified, ligated and electroporated as described (35).

CFE experiments and coimmunoprecipitation*:* The HindIII/EcoRI (filled-in) fragment, encoding the full spinophilin, derived by pGEM-Spinophilin was cloned in pRcCMV (3) cut by HindIII/NotI (filled-in) (pCMV-Spinophilin). The fragment HindIII (filled-in)/XbaI was excised from pCMV-Spinophilin and cloned in pcDNA3-HisA (CLONTECH, UK) cut by EcoRI (filled-in)/XbaI (pcDNA-Spinophilin). EcoRI/SalI fragment derived from pBTM-ARF was also cloned in pcDNA3-HisA (pcDNA-ARF). To obtain the N-terminal region of

spinophilin (SpinoN) encoding aa 1-473 the pCMV-Spinophilin was cut by BamHI and HindIII (filled-in). The SpinoN fragment was cloned in pcDNA3-HisA cut by EcoRI (filled-in)/BamHI (pcDNA-SpinoN). The C-terminal region of spinophilin (SpinoC) encoding aa 605-813 was obtained by EcoRI digestion of pGAD10-SpinoC and cloned in pcDNA3-HisA cut by EcoRI (pcDNA-SpinoC). PGAD-nH-AB, pGAD-nH-A, pGAD-nH were cut by EcoRI/XbaI and the DNAs encoding respectively 605-787, aa 605-728 and 605-668 were cloned in pcDNA3-HisA cut by EcoRI/XbaI to generate pcDNA-nH-AB, pcDNA-nH-A and pcDNA-nH. The human MDM2 (HDM2) in pcDNA3-His was a kind gift of B. Vogelstein.

Cellular localization experiments: The HindIII/EcoRI fragment excised from pGEM-Spinophilin was cloned in pEGFP-C2 cut with the same restriction enzymes (pEGFP-Spinophilin). EcoRI/SalI fragment, excised from pBTM-ARF, was cloned in pEGFP-C2 (pEGFP-ARF). pEGFP-SpinoN was obtained cloning the HindIII/BamHI fragment, encoding the amino acids 1-473 of spinophilin, in pEGFP-C2 cut with the same restriction enzymes. To obtain pEGFP-SpinoC encoding the amino acids 552-813 of spinophilin, the HindIII/BglII fragment (amino acids1-813), excised from pCMV-Spinophilin was cloned in pEGFP-C3 cut with HindIII/BamHI. Subsequently pEGFP-Spinophilin was cut with BglII/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 (Pharmacia, S) to obtain pGEX-SpinoC plasmid. EcoRI/SalI fragment excised from p19 plasmid was cloned in pMAL-c2 (Biolabs, UK) 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 construct was used to screen a human brain cDNA library cloned into the pGAD10 vector (Clontech, UK). 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 confirmed by β-galactosidase activity. Isolated plasmids were retransformed into L40 with the negative control plasmid pBTM-galactin (a gift of L. Chiariotti) 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 (TM) database using the BLAST search algorithm (37) available at www.ncbi.nlm.nih.gov.

Anti-ARF antibody preparation and purification ↑ The MBP::ARF fusion protein was obtained by expression in TG1 *E. coli* strain transformed with pMAL-ARF plasmid and purified on amylose-agarose (Pharmacia, S) following the procedure suggested by the manufacturer. The protein was further purified by gel-filtration on S-300 (Pharmacia, S). Anti-ARF polyclonal antibodies were raised in rabbit using purified MBP::ARF fusion protein. Antibodies were purified by caprilic acid precipitation (38). To remove anti-MBP antibodies, the antibodies were subsequently loaded on MBP coupled to sepharose 4B (Pharmacia, S). The anti-ARF antibodies were finally purified and concentrated on proteinA-sepharose (Pharmacia, S) following the procedure suggested by the manufacturer. Anti-ARF antibodies show a detection limit of 5 ng and 50 ng respectively for ARF and MBP (data not shown).

ARF, spinophilin and PP1c in vitro interaction ↑ The GST::SpinoC fusion protein was obtained by expression in TG1 *E. coli* strain transformed with pGEX-SpinoC plasmid and

purified by affinity chromatography on glutathione sepharose 4B (Pharmacia, S) followed by gel-filtration, in PBS buffer, on Superdex 200 HR (Pharmacia, S). MBP and MBP::ARF were expressed in *E. coli* and purified by affinity chromatography on amylose-agarose (Biolabs, UK) followed by gel-filtration, in PBS buffer, on S-300 (Pharmacia, S). Approximately 3 μg of purified MBP or MBP::ARF were mixed with the same amount of GST or GST::SpinoC in TENN buffer (50 μl, 50 mM Tris HCl pH 7.4, 1 mM EDTA pH 8, 10% NaCl, 0.1% NP 40, 1mM DTT, 1 mM PMSF, 1% BSA) and (25 μl gel volume) amylose-agarose (Biolabs, UK) was added (20 minutes at 4°C). The beads were collected by centrifugation, washed three times with TENN buffer containing 1 M urea and twice with TENN buffer. Samples were loaded on 10% SDS-PAGE, blotted on nitrocellulose paper (Schleicher & Schuell) and probed with anti-GST antibody (1:2000; Sigma, USA) followed by horseradish peroxidase (HRP) anti-goat immunoglobulin G (1:1000; Sigma, USA). Immunoreactive bands were visualized by enhanced chemioluminescence (Amersham, UK).

His6::Xpress::PP1c was obtained by expression in TOP10 *E. coli* strain (Invitrogen) transformed with pBAD-PP1c plasmid. 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 hour at 37°C. Filters were separately incubated with GST::SpinoC and GST (50 μg/ml, 18 hours, 4°C), followed by anti-GST polyclonal antibody (1:2000, 2 hours, 37°C) and by HRP-anti-goat immunoglobulin G (1:1000, 2 hours, 37°C). Immunoreactive bands were visualized by enhanced chemioluminescence.

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 (DMEM) supplemented with 2mM glutamine, penicillin/streptomycin 100U/ml each and 10% fetal bovine serum (FBS) except for NIH3T3 cultured in the same medium supplemented with 10% (CS) calf serum.

For coimmunoprecipitation assay, COS-7 cells were seeded into a 6-well multiplate ($2{,}5 \times 10^5$ cells per well) and transfected using the Superfect reagent according to the manufacturer's instruction (Qiagen). Cells were washed twice with ice-cold PBS, lysed into NP-40 lysis buffer (50 mM NaCl, 150 mM Tris-HCl, 1% NP-40, 0.5% Sodium deoxicholate, 10% glycerol, 1 mM PMSF and protease inhibitor). The lysate was passed through a 21 gauge needle, debris were removed by centrifugation (13,000 x g, 10 minutes, 4°C) and the total amount of proteins was quantified (Biorad Protein assay). Equal amount of lysates were pre-cleared with protein A sepharose beads (Repligen, UK; 8 hours at 4°C) and subsequently incubated with anti-ARF antibody (dilution 1:100, 2 hours at 4°C) followed by addition of protein A sepharose beads (50 µl). Beads were collected by centrifugation, the immunoprecipitates were washed three times with lysis buffer (4°C), solubilized in SDS-PAGE sample buffer, loaded on 8% SDS-PAGE and analyzed by immunoblotting with anti-Xpress antibody (1:2000, 3 hours, at room temperature; Invitrogen) followed by incubation with the HRP-anti mouse antibody (1:1000, 1 hour, at room temperature; Amersham, UK). Immunoreactive bands were visualized by enhanced chemioluminescence.

For imaging analysis NIH3T3 and COS-7 cells were grown on a sterilized glass coverslip (5×10^4 cells per slip) into 6-well multiplates and transfected using the Superfect reagent (Qiagen) according to the manufacturer's instructions. Twenty-four hours after transfection, cells were washed twice with PBS and fixed with 4% para-formaldehyde in PBS (30

minutes). Glass coverslips were subsequently washed twice with PBS and mounted on microscope slides with 50% glycerol in PBS. Images were acquired using a confocal microscope Axiovert 100M Zeiss. A krypton-argon gas laser provided excitation at 488 nm with a 522/32 emission filter for green fluorescence.

For the CFE assay (colony formation efficiency assay) U2OS, Saos-2 ($1x10^5$ per well) and NIH3T3 ($2x10^4$ per well) cells were seeded into 6-well multiplates and transfected with 2 μg of total plasmid DNA using the standard calcium phosphate method (39). Forty-eight hours after transfection the cells were replated in a 100 mm dish and selected with the appropriate concentration of Geneticin (G418, Calbiochem, USA) for two weeks. Saos-2 and NIH3T3 cells were selected with 1 mg/ml of G418 and U2OS cells with 600 μg/ml of G418. Medium was replenished every three days. Cells were fixed with 10%methanol/10%acetic acid and stained with crystal violet 15 days post-transfection. The colonies were counted. Each experiment was repeated at least 4 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 (36). 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 approximately 10^7 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 database 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, 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 database 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 a 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)XF (grey triangle), a PDZ domain and a myosin-like left handed alpha-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 acids 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. 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 spinophilin, 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, as the complexes were not found when either protein alone was introduced into the cells (Fig. 2C, compare, lanes 3, 4 to lanes 5, 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 the 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 rod), helix A (674-726, white rectangle), helix B (727-788, black rectangle) and helix C (789-813, grey rectangle). 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 while 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 coimmunuprecipitate with ARF in cell extracts. COS-7 cells were transfected with mammalian expression plasmids pcDNA-nH-AB (aa 605-787), pcDNA-nH-A (aa 605-728) and pcDNA-nH (aa 605-668) and/or pcDNA-ARF. As shown in Fig. 3B (lanes 5-7) only the shorter mutant failed to interact with ARF, suggesting again a key role for helix A in the contact between spinophilin and ARF.

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-X-F) 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.

As ectopic expression of ARF results in growth suppression, in order 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) while 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-418^R colony formation only in the U2OS and NIH3T3 cell lines, that 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-418^R 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.

We have also analyzed the effect of cotransfection of the two plasmids and the results show that simultaneous expression of both proteins in human cells resulted in a stronger effect on inhibition of colony formation. The data in U2OS cells can be explained as the result of an additive effect of the two proteins. In Saos-2 cells, however, transfection of 1μg each of spinophilin 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) Lin-10 (42). ARF has a preferential localization in the nucleolus, but, when overexpressed with MDM2 and p53, it has also been found in "nuclear bodies" within the nucleoplasm (16). As 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. In order 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. 5, panel C). 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, as this region is deleted in the GFP::SpinoN mutant (1-473) which is clearly excluded from the nucleoplasm (Fig. 5, panel E). 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. 5, panel D).

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 (our unpublished results) 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, as 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 portions interacting with spinophilin and MDM2 are partially overlapping (43). We have also shown that the PDZ and the other protein/protein interaction domains present in the N-terminal region of spinophilin are not involved in the contact with ARF as, instead, only part of the coiled-coil region present in the C-terminal region of spinophilin (aa. 605-726) is required in yeast and in mammalian cells for an efficient interaction.

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 (39-42). However, in our localization experiments we were able to demonstrate that, at least in the cell types we used (COS-7 and NIH3T3), spinophilin localizes both in the nucleoplasm and in the cytoplasm, and that deletion of the C-terminal portion of the protein resulted in the exclusion of the protein from the nucleus. On the other hand, the analysis of spinophilin with the psort server (psort.nibb.ac.jp), using Reinhardt's method for Cytoplasmic/Nuclear discrimination (44)

suggested an 89% probability of nuclear localization although the program failed to identify canonical nuclear localization signals. It is possible that subcellular compartmentalization of spinophilin could in some way be tissue-specific, and/or could depend, at least in part, on interaction with different partners. Colocalization experiments between ARF and spinophilin will help to clarify this point.

Protein phosphorylation and dephosphorylation regulates many cellular functions. Protein kinases and phosphatases have multiple substrates *in vivo*, which enables several responses to physiological stimuli. However their broad substrate specificity suggests the need for mechanisms to restrict the action of these enzymes *in vivo*. Recent evidence reported that some protein phosphatases and kinases are regulated by targeting subunits (45). This class of protein not only acts to 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, as 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 (35)) and it has been implicated in the mitotic dephosphorylation of pRb (35), as well as in the dephosphorylation of specific residues of p53 (35,46). PP1 is found associated with pRb in the G1 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. Mitotic 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 (53). Potential targets for PP1 in the nucleus are the mitotic cyclin B/cdk1 substrates that include histone H1, lamins, 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 inhibited growth of G418 resistant colonies up to 50% respect to the control, suggesting that it could act in a pathway that is at least in part, p53-independent. Moreover, as coexpression resulted in a remarkable level of inhibition of colony formation, it might be speculated that ARF enforced the activity of spinophilin or, vice versa, expression of spinophilin activated a p53 independent ARF activity. Interestingly, it has been very recently reported (28) that triple knock out mice nullizygous for ARF, p53 and MDM2 (TK0) develop multiple tumors at a frequency greater than those observed in animals lacking both p53 and MDM2 or p53 alone, demonstrating that ARF can act independently of the MDM2-p53 axis in tumor surveillance. Moreover, reintroduction of ARF into TK0 mouse embryo fibroblasts arrested the cell division in the G1 phase. The authors conclude that in absence of MDM2 and p53, ARF interacts with other unknown proteins to inhibit cell proliferation. Thus, it might be possible that only in a p53 null context could overlapping

pathways become unmasked.

In order to clarify these points, further experiments are necessary, in particular to elucidate whether a cell cycle arrest or the activation of apoptotic or senescence pathways is responsible for inhibition of proliferation induced by spinophilin.

Our data also indicate that the ARF-binding domain of the spinophilin does not have any effect on cell growth, regardless of the status of endogenous ARF. Moreover cotransfection of ARF-binding domain of the spinophilin and ARF again does not result in any effect on the biological activity of ARF.

In conclusion our experiments suggest that the physical interaction between human oncosuppressor ARF and a PP1-binding protein might result in a functional interaction, depending on the genetic context. In particular, the results in Saos-2 cells let us to suppose that ARF and spinophilin could act in partly overlapping pathways. One possible scenario is that this interaction could function to target PP1 on ARF and/or on other molecules involved in the same pathway. We are now investigating the possibility that phosphorylation and dephosphorylation could play a role in the regulation of ARF biological activity.

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Figures legend:

Figure 1: A) schematic illustration of full-length human spinophilin/neurabin-II (817 aa) and of the isolated clones (B2 and SpinoC). Indicated are the position of the F-actin-binding domain (ABD), the PP1c binding site (grey triangle), the PSD-95/discs large/ZO-1 (PDZ) domain, and the predicted coiled-coil motif at the C-terminal region. B) Comparison between the human and the rat sequence (amino acids substitutions only are indicated). The ABD domain is almost identical between the human and rat (underlined sequence) showing 96% amino acids identity. The PP1c binding site, (bold sequence) as the PDZ domains, (bold italics sequence) are 100% identical to each other. The coiled-coil motif at the C-terminal region shows an amino acids identity of 98% between human and rat. The human spinophilin sequence was deposited in EMBL with the accession numbers AJ401189, HSA401189.

Figure 2: In vitro/in vivo interaction between ARF and spinophilin. A) Co-precipitation experiments were performed using purified proteins. Approximately 3 μg of MBP::ARF or MBP were mixed with the same amount of GST::SpinoC or GST and amylose-agarose was added. The beads were washed and the samples analyzed by immunoblotting with anti-GST antibody. MBP::ARF coprecipitates efficiently GST::SpinoC (lane 1) but not GST (lane 2). Furthermore, MBP does not coprecipitate GST::SpinoC (lane 3). The correct size of GST::SpinoC fusion protein is defined by an aliquot of GST::SpinoC purified protein (lane 4) B) Far-western: Two aliquots of MBP::ARF were loaded on 10% SDS-PAGE, blotted on nitrocellulose paper and separately probed with either GST::SpinoC or GST protein. Filters were subsequently incubated with anti-GST antibody. MBP::ARF binds only to GST::SpinoC (lane 1), while no interaction was observed using as tracer GST (lane 2). C) Co-immunoprecipitation of full length spinophilin with ARF: 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. The full length spinophilin and MDM2 were detected after co-immuno-precipitation only when ARF was coexpressed (compare lanes 3, 4 to lanes 5 and 6). D) Co-immunoprecipitation of spinophilin C-terminal domain with ARF: COS-7 cells were transfected with mammalian expression plasmids encoding Xpress-tagged spinophilin or

Xpress-tagged SpinoN (1-472) or Xpress-tagged SpinoC (aa 605-813), 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 coimmunoprecipitation when ARF was coexpressed (lanes 6 and 7).

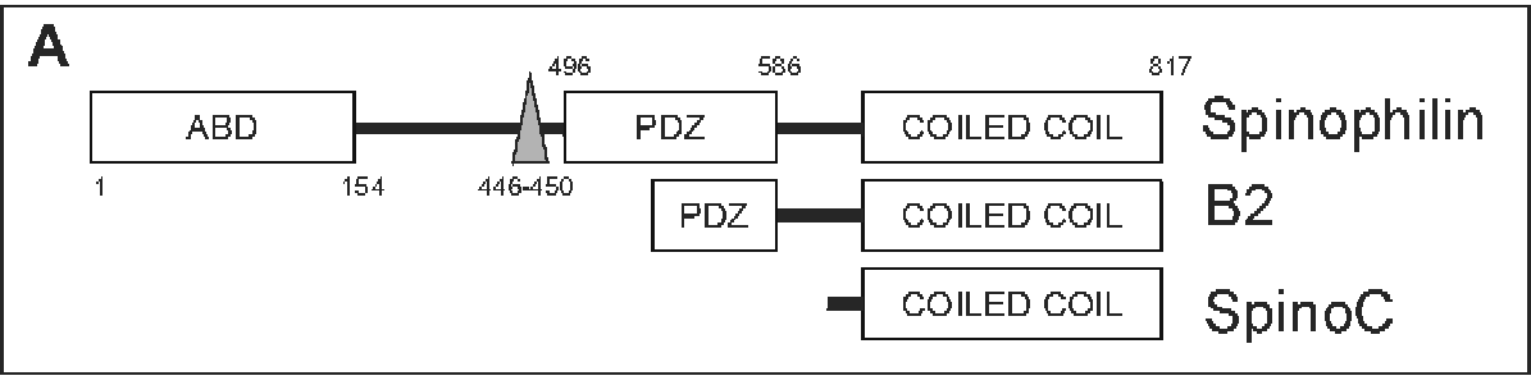
Figure 3: Mapping the interaction domains between ARF and spinophilin. Expression vectors encoding ARF, the deletion mutants ARF-(1-65), ARF-(1-27) and ARF-(38-132) were probed in the yeast two hybrid system for interaction with expression vector encoding the C-terminal part of spinophilin (aa 605-813, lanes a-d). Expression vectors encoding respectively amino acids 605-787 (nH-AB), 605-728 (nH-A) and 605-668 (nH) of spinophilin were probed in yeast two hybrid system for interaction with the ARF-(1-65) (lanes e-g). Interaction between ARF and spinophilin deletion mutants was qualitatively identified by histidine auxotrophy (HIS-growth panel) and quantitatively by β-galactosidase activity measurement (β↑gal activity panel). Values represent the means of three independent experiments. Standard deviation for each value is also given. B) Coimmunoprecipitation of C-terminal deletion mutants of spinophilin with ARF: COS-7 cells were transfected with mammalian expression plasmids pcDNA-nH-AB (aa 605-787), pcDNA-nH-A (aa 605-728) and pcDNA-nH (aa 605-668) and/or pcDNA-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 deletion mutants without the co-expression of ARF (Lane 2-4). Only the mutants pcDNA-nH-AB (aa 605-787) (lane 7) and pcDNA-nH-A (aa 605-728) (lane 6) could be detected after coimmunoprecipitation in presence of ARF.

Figure 4: Growth suppression by ARF and spinophilin expression. Saos-2, U2OS and NIH3T3 cell lines were seeded into 6-well multiplates and transfected with the indicated amounts of ARF and spinophilin expression vectors. Forty-eight hours after transfection the cells were replated in a 100 mm dish and selected with the appropriate concentration of G418 for two weeks. The cells were fixed and stained with crystal violet, and the colonies were

counted. The graphic represents the percentage of colonies obtained with the indicated plasmids relative to that detected on the pcDNA3 transfected plates. Values represent the mean of four independent experiments. Standard deviation for each value is also given.

Figure 5: Subcellular localizations. COS 7 and NIH3T3 cells were seeded on glass coverslips and transfected with expression vectors encoding respectively the fusion proteins GFP::ARF (panel A), GFP (panel B), GFP::spinophilin (panel C), GFP::spinoC (panel D) and GFP::spinoN (panel E). Cells were fixed twenty-four hours after trasfection and images were acquired using a confocal microscope Axiovert 100M Zeiss.

Fig. 1


A
496
586
817
ABD
PDZ
COILED COIL
Spinophilin
1
154
446-450
PDZ
COILED COIL
B2
COILED COIL
SpinoC

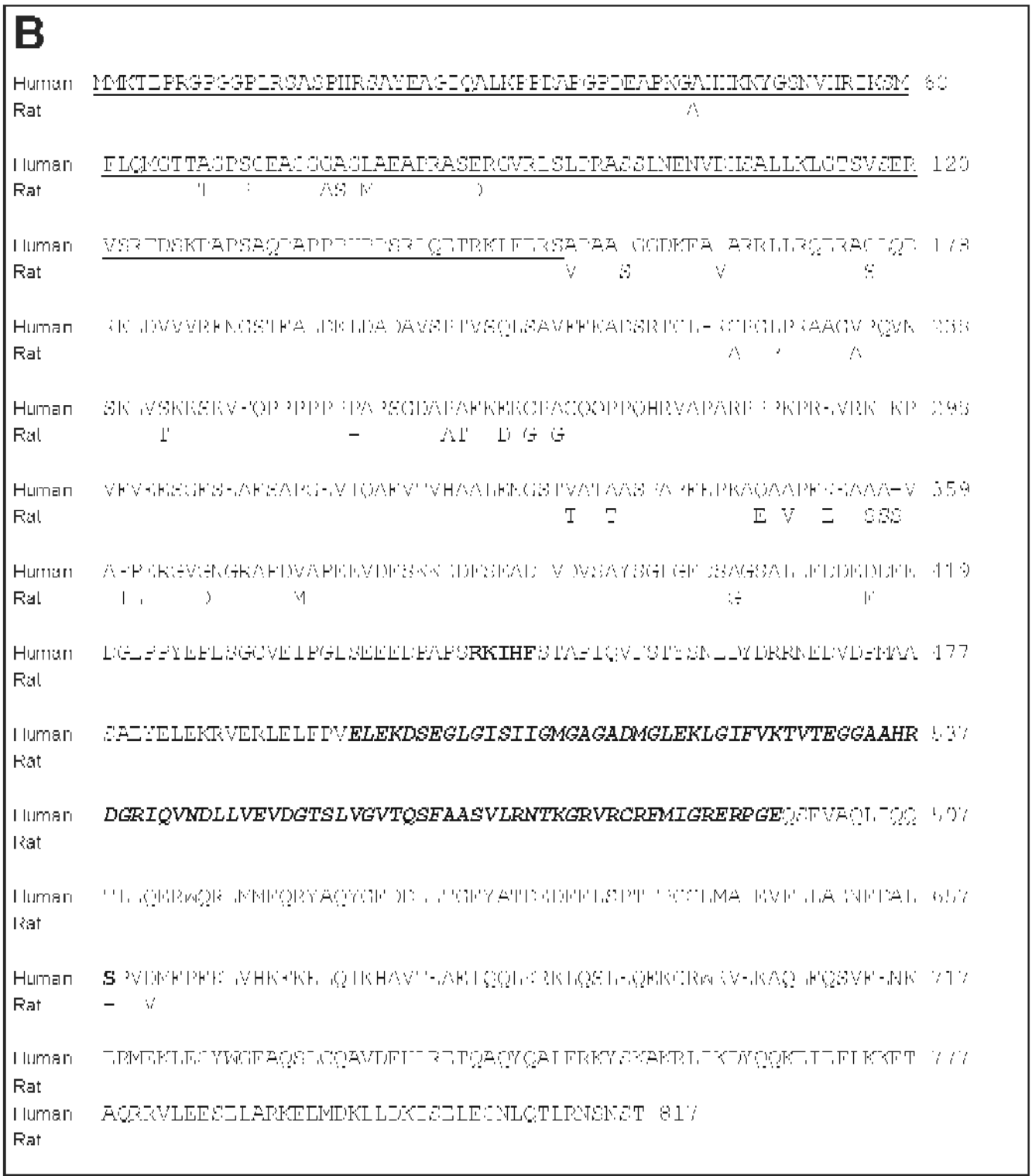
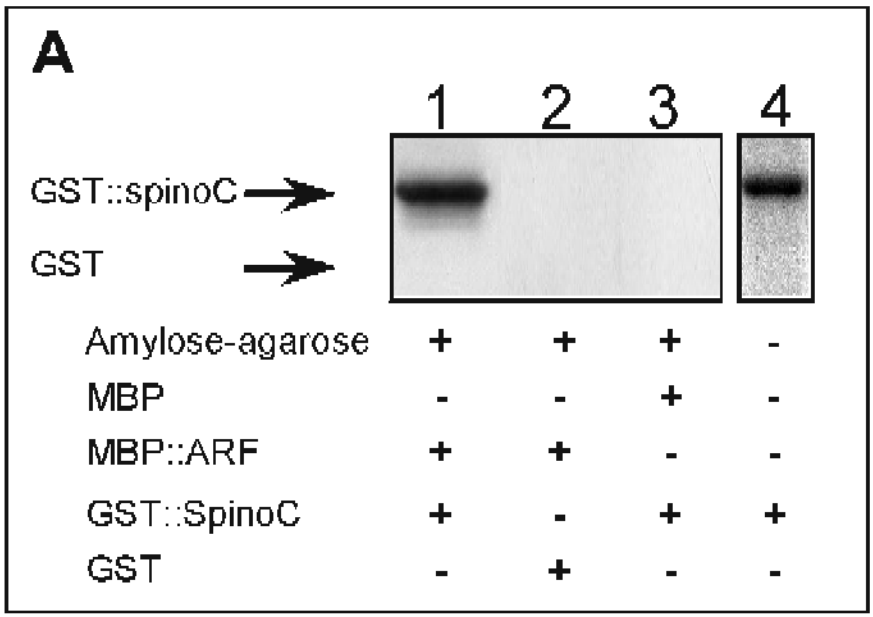
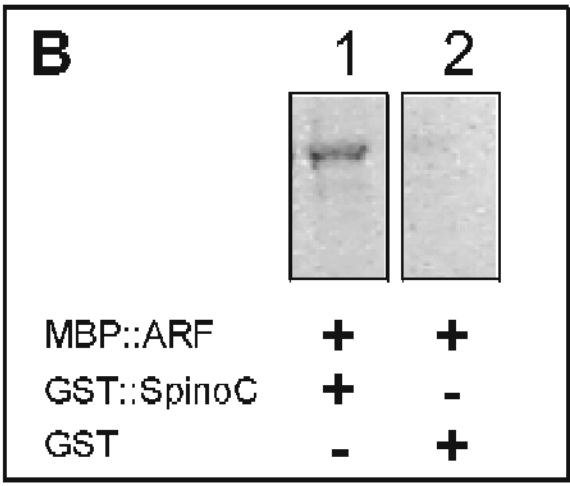
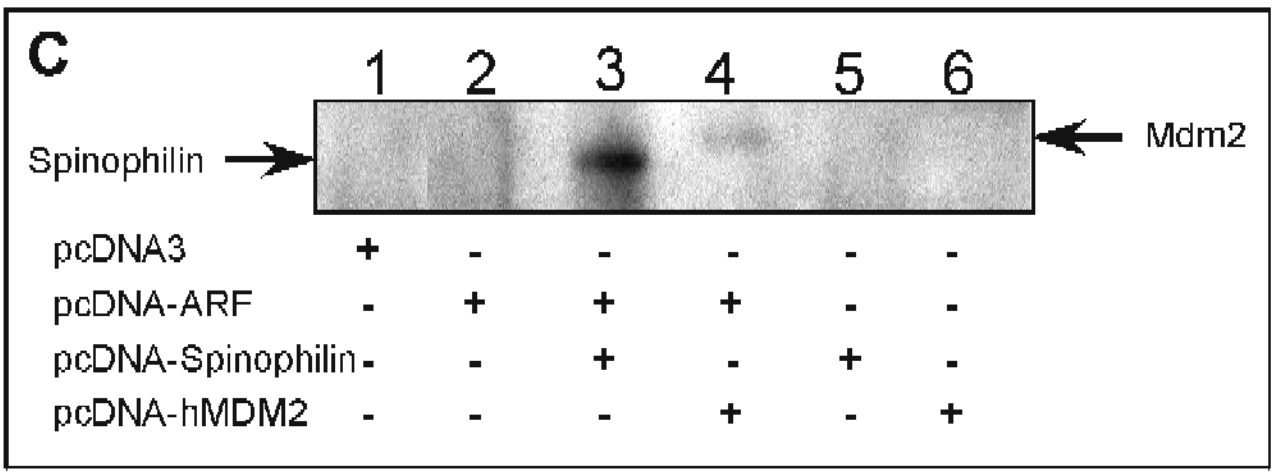

B
Human
Rat

Fig. 2


A
1 2 3 4
GST::spinoC
GST
Amylose-agarose + + + -
MBP - - + -
MBP::ARF + + - -
GST::SpinoC + - + +
GST - + - -


B
1 2
MBP::ARF + +
GST::SpinoC + -
GST - +


C
1 2 3 4 5 6
Spinophilin
Mdm2
pcDNA3 + - - - - -
pcDNA-ARF - + + + - -
pcDNA-Spinophilin - - + - + -
pcDNA-hMDM2 - - - + - +

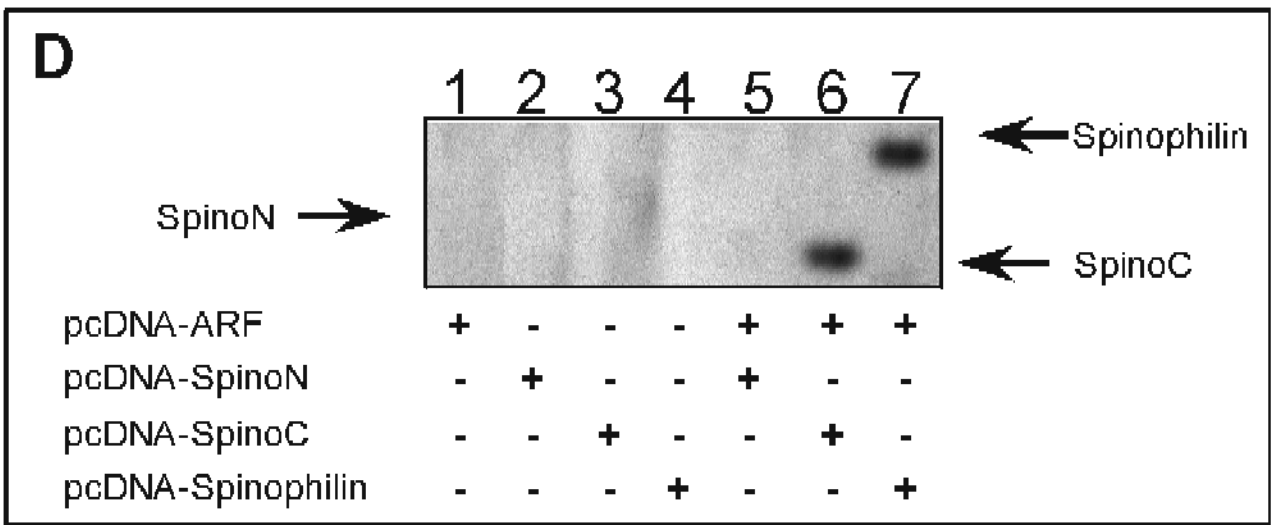

D
1 2 3 4 5 6 7
SpinoN
Spinophilin
SpinoC
pcDNA-ARF + - - - + + +
pcDNA-SpinoN - + - - + - -
pcDNA-SpinoC - - + - - + -
pcDNA-Spinophilin - - - + - - +

Fig. 3

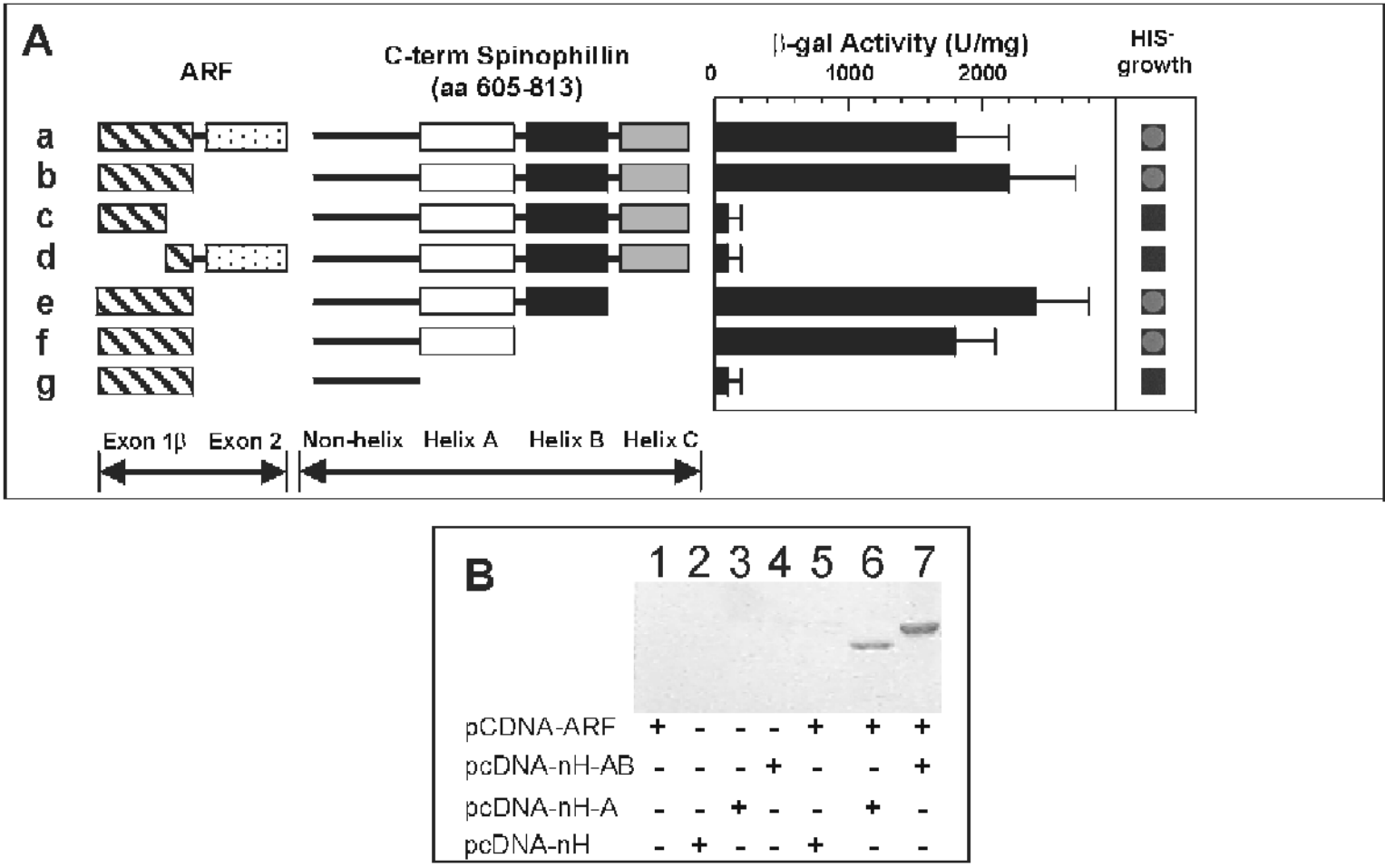

A
ARF
C-term Spinophillin
(aa 605-813)
β-gal Activity (U/mg)
0
1000
2000
HIS⁻
growth
a
b
c
d
e
f
g
Exon 1β
Exon 2
Non-helix
Helix A
Helix B
Helix C
B
1 2 3 4 5 6 7
pCDNA-ARF + - - - + + +
pcDNA-nH-AB - - - + - - +
pcDNA-nH-A - - + - - + -
pcDNA-nH - + - - + - -

Fig. 4

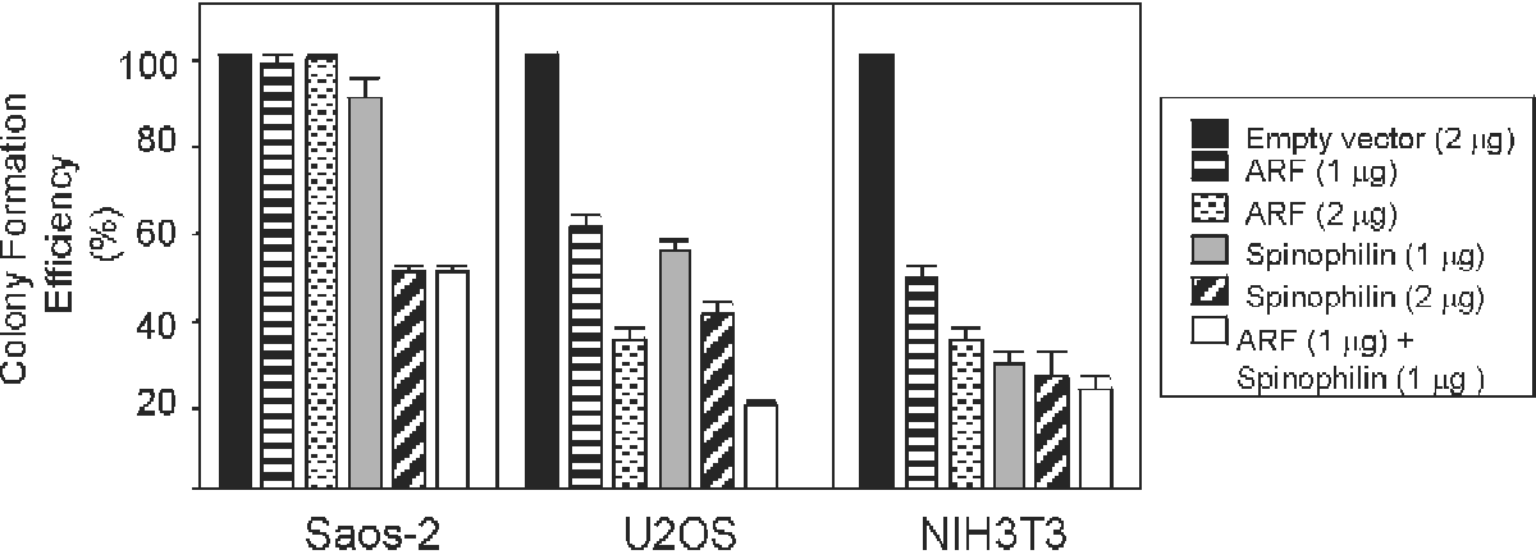
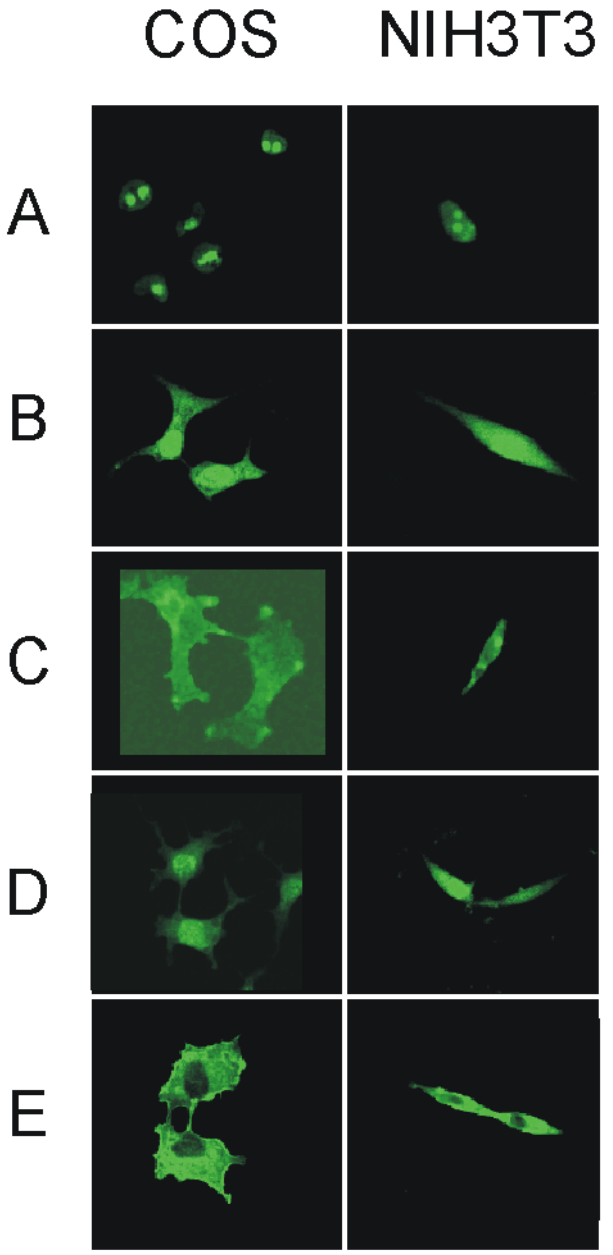

Colony Formation Efficiency (%)
100
80
60
40
20
Saos-2
U2OS
NIH3T3
Empty vector (2 μg)
ARF (1 μg)
ARF (2 μg)
Spinophilin (1 μg)
Spinophilin (2 μg)
ARF (1 μg) + Spinophilin (1 μg)

Fig. 5



The human tumor suppressor ARF interacts with spinophilin/neurabin II, a type 1 protein-phosphatase-binding protein

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