A developmentally regulated ARF-like 5 protein (ARL5), localized to nuclei and nucleoli, interacts with heterochromatin protein 1

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Summary

ARF-like proteins (ARLs) are distinct group of members of the ARF family of Ras-related GTPases. Although ARLs are very similar in primary structure to ARFs, their functions remain unclear. We cloned mouse (m) and human (h) ARL5 cDNAs to characterize the protein products and their molecular properties. mARL5 mRNA was more abundant in liver than in other adult tissues tested. mARL5, similar to mARL4, was developmentally regulated and localized to nuclei. hARL5 interacted with importin-α through its C-terminal bipartite nuclear localization signal. When expressed in COS-7 cells, mutant hARL5(T35N), which is predicted to be GDP bound, was concentrated in nucleoli. The N-terminus of hARL5, like that of ARF, was myristoylated. Yeast two-hybrid screening and in vitro protein-interaction assays showed that hARL5(Q80L), predicted to be GTP bound, interacted with heterochromatin protein 1α (HP1α), which is known to be associated with telomeres as well as with heterochromatin, and acted as a transcriptional suppressor in mammalian cells. The interaction was reproduced in COS cells, where hARL5(Q80L) was co-immunoprecipitated with HP1α. hARL5 interaction with HP1α was dependent on the nucleotide bound, and required the MIR-like motif. Moreover, hARL5(Q80L), but not hARL5 lacking the MIR-like motif, was partly co-localized with overexpressed HP1α. Our findings suggest that developmentally regulated ARL5, with its distinctive nuclear/nucleolar localization and interaction with HP1α, may play a role(s) in nuclear dynamics and/or signaling cascades during embryonic development.

Key words: ADP-ribosylation factors, Myristoylation, Heterochromatin, Importin-α, HP1α, Embryonic development

Introduction

ADP-ribosylation factors (ARFs) are a family of highly conserved, ~20 kDa guanine nucleotide-binding proteins that have important roles in vesicular transport (reviewed by Boman and Kahn, 1995; Moss and Vaughan, 1998). ARFs regulate membrane traffic and the actin cytoskeleton, and directly activate phospholipase D and phosphatidylinositol 4-phosphate 5-kinase. The ARF family comprises at least six ARFs and six ARF-like (ARL) proteins (Boman and Kahn, 1995; Moss and Vaughan, 1998). Both ARFs and ARLs are widely distributed in eukaryotic organisms from yeast to human, consistent with evolutionary conservation of their biological functions. ARLs are structurally divergent, with sequences 40-60% identical to any ARF or to each other. ARLs, unlike ARFs, usually do not activate cholera toxin ADP-ribosyltransferase, although hARL1 had relatively low activity (Hong et al., 1998). ARLs differ also from ARFs in having demonstrable GTPase activity and different conditions that favor guanine nucleotide binding (Moss and Vaughan, 1998).

The biological functions of ARLs are still unclear, although some are expressed in tissue- and/or differentiation-specific patterns (Clark et al., 1993; Cavenagh et al., 1994; Schurmann et al., 1994; Zhang et al., 1995; Hong et al., 1998). ARLl was localized in the Golgi complex of normal rat kidney cells (Lowe et al., 1996) and *S. cerevisiae* (Lee et al., 1997), consistent with a function in vesicular trafficking. Unlike the lethal phenotype of double null alleles of *arf1* and *arf2*, however, knock-out of the yeast ARL1 gene was not lethal (Lee et al., 1997). Expression of ARL4 was reported to be cell differentiation-dependent and developmentally regulated (Clark et al., 1993; Lin et al., 2000). ARL4, with its distinctive nuclear/nucleolar localization and pattern of developmental expression, was inferred to play a unique role(s) in neurogenesis and somitogenesis during embryonic development and in the early stages of spermatogenesis in adults (Lin et al., 2000).

To obtain additional clues to physiological role(s) of ARLs, we investigated the expression, subcellular localization, and biochemical properties of ARL5. As reported here, expression of mouse ARL5 (mARL5), similar to that of mARL4, was developmentally regulated during embryogenesis and was mainly detected in nuclei. When expressed in COS-7 cells, hARL5(T35N), a mutant predicted to be GDP-bound, was localized to nucleoli. Data from yeast two-hybrid and protein interaction analyses revealed that hARL5 interacted with the heterochromatin protein 1α (HP1α, through its C-terminal MIR-like motif and this interaction was nucleotide-dependent.

HP1α is one of the three mammalian HP1 family proteins that have been found in many other organisms from *Schizosacchromyces pombe* (Lorentz et al., 1994) to mammals (Singh et al., 1991; Saunders et al., 1993). HP1α is a nonhistone chromosomal protein suppressor of position effect variegation in *Drosophila* (James and Elgin, 1986; Eissenberg et al., 1990). It is associated with heterochromatin (James and Elgin, 1986; Eissenberg et al., 1990) and telomeres (Fanti et al., 1998), and prevents telomere fusion (Fanti et al., 1998). Thus, ARL5, like ARL4, may have a physiological role(s) in nuclear dynamics and/or signaling cascades during embryonic development.

Materials and Methods

Isolation of mouse and human ARL5 cDNA

Mouse ARL5 cDNA was synthesized by polymerase chain reaction (PCR) from a mouse λgt11 cDNA library. PCR-based cloning methods were used to obtain cDNA segments, from which a composite sequence of the full-length coding region was assembled (Lee et al., 1994). A probe composed of degenerate oligonucleotides (ARL5-R1, and ARL5-R2) (Table 1) corresponding to part of the consensus sequences WDVGGQE and KLRPLWR in human and mouse ARL4 was used to screen a mouse λgt11 cDNA library (Clontech) in the one-site-specific PCR to capture 3′ and 5′ ends of mouse ARL5 cDNA as previously described (Lee et al., 1997). All PCR products were purified, subcloned, and sequenced by the dideoxy chain-termination method (Sanger et al., 1977). The nucleotide sequence of mouse ARL5 has GenBank accession number AF312686. Human ARL5 cDNA was isolated by same procedures. A nucleotide sequence identical to that of hARL5 was deposited by Smith et al. (Smith et al., 1995) (GenBank accession number U25771).

Full-length wild type hARL5 cDNA was generated using 5′ (sense) primer ARL5A, and 3′ (antisense) primer ARL5B (Table 1). Replacement of Thr35 with Asn (T35N), Gln80 with Leu (Q80L), and deletion of the MIR motif of ARL5 were accomplished using a two step PCR technique as described (Lee et al., 1997). The 5′ (sense) mutagenic primer ARL5C and the antisense mutagenic primer ARL5D were used to generate hARL5(T35N). The 5′ (sense) mutagenic primer ARL5E and the antisense mutagenic primer ARL5F were used to generate hARL5(Q80L). The point mutation is underlined in oligonucleotide sequences (Table 1). To generate ARL5(dMIR) (MIR-deleted mutant) with deletion of 6 amino acids (positions 128 through 133) that include the MIR motif, the 5′ (sense) primer ARL5G and the antisense primer ARL5H were used. To generate ARL5(dC) with deletion of 24 C-terminal amino acids (positions 176 through 201) that include the putative nuclear localization signal (NLS), hARL5 cDNA clone was digested with *Eco*NI and *Xba*I, blunt-ended, and ligated.

Northern analyses

Blots with RNAs from adult mice and mouse embryos at several stages of development (Clontech) were processed for hybridization with mARL5-specific probes as described previously (Lee et al., 1994).

Expression and purification of recombinant proteins

The entire open reading frame of human ARL5 was obtained by PCR, using primers that incorporated unique *Nde*I and *Bam*HI sites, respectively, at the initiating methionine and six bp downstream from the stop codon. For preparation of the His-tagged fusion protein, the hARL5 PCR product was cloned into the expression vector pET15b (Novagen), yielding pET15b-His-hARL5, which was used to transform BL21 (DE3) (Lee et al., 1997). Cell pellets were harvested and His-tagged fusion protein was isolated on Ni^{2+}-NTA resin (Qiagen, Chatsworth, CA) by standard methods. The purity of the His-tagged hARL5 was assessed by SDS-PAGE.

Generation of ARL5 antisera and immuno-analyses

Rabbits were immunized with keyhole limpet hemocyanin-conjugated synthetic peptide GNHLTEMAPTASSFLPC (peptide N), corresponding to the residues 2-18 of hARL5. Antibodies (ARL5-N) were affinity-purified on immobilized, recombinant hARL5. Western analysis and immunoprecipitation were performed according to the procedures of Harlow and Lane (Harlow and Lane, 1988).

Fractionation by differential centrifugation

Nuclear (N), crude cytosol (C), and membrane (M) fractions were prepared as described previously (Schreiber et al., 1989; Yang et al., 1998). Briefly, confluent cells were scraped and homogenized in HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 250 mM sucrose) plus 1 mM phenylmethylsulfonyl fluoride (PMSF) and a mixture of

Table 1. Oligonucleotides used in the analysis of ARL5

Name	Sequence (5′→3′)
ARL-R1	T/C-T-C-T/C-T-G-A/C/G/T- C-C-ACGT-C-C-A/C/G/T-A-C-A/G-T-C-C-C-A
ARL-R2	T/C-T-T-C-C-A-A/C/G/T-A-A/G-A/C/G/T-G-G-A/C/G/T-C-G/T-A/C/G/T-A-A/G-C/T-T-T
ARL5A	CAT ATG GGG AAC CAC TTG ACT GAG ATG*
ARL5B	TCT AGA CCG TCT CTT CTT GCC ACC C†
ARL5C	TG GAC TCT GCT GGA AAG AAC TCC CTC CTT TAC CGC CT‡
ARL5D	AG GCG GTA AAG GAG GGA GTT CTT TCC AGC AGA GTC CA‡
ARL5E	TG TGG GAC GTC GGG GGG CTG GAG AAG CTG CGA CCA CT§
ARL5F	AG TGG TCG CAG CTT CTC CAG CCC CCC GAC GTC CCA CA§
ARL5G	TCG GAC AAC CAG GGC GCC AAC AAG CAG GAC CAG¶
ARL5H	GTC CTG CTT GTT GGC GCC CTG GTT GTC CGA GGC¶
ARL5I	CAT ATG GCG AAC CAC TTG ACT GAG ATG**
ARL5J	G AAT TCC ACC ATG GGG AAT GGG CTG TCA GAC CAG††

*Underlined bases introduce *Nde*I site.
†Underlined bases introduce *Xba*I site.
‡Underlined bases introduced T35N point mutation.
§Underlined base introduces Q80L point mutation.
¶MIR motif deletion.
**Underlined base introduces G2A point mutation.
††Underlined bases introduce *Eco*RI site, for primer used to insert into pBTM116.

protease inhibitors (leupeptin, aprotinin, chymostatin, antipain, and pepstatin, each 1 μg/ml) at 4°C by 10 strokes in a ball-bearing homogenizer. The cell lysate was centrifuged at 400 ***g*** for 10 minutes to sediment unbroken cells, nuclei, and cell debris. The supernatant was centrifuged (150,000 ***g***, 1 hour) at 4°C to generate cytosolic (C) and membrane (M) fractions. To obtain the nuclear fraction, cell pellet containing unbroken cells, nuclei, and cell debris was dispersed in 1 ml of TBS (Tris-buffered saline), transferred to an Eppendorf tube and centrifuged for 15 seconds in a microfuge. TBS was removed and the pellet was suspended in 400 μl of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) by gentle pipetting in a micro-pipette tip. The cells were allowed to swell on ice for 15 minutes, after which 25 μl of a solution containing 10% Nonidet P-40 were added and the tube was vigorously vortexed for 10 seconds. The homogenate was centrifuged for 30 seconds in a microfuge, and the nuclear pellet (N) was collected (Schreiber et al., 1989). Equivalent amounts of the nuclear, cytosol and membrane fractions were analyzed by immunoblotting analysis.

Cell culture and transient transfection

COS-7 cells (ATCC: CRL-1651) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10% fetal bovine serum, 2 mM glutamine and 100 units/ml each of penicillin and streptomycin. The Hep 3B cell-line (ATCC: HB-8064) was grown in the same medium with additional 0.1 mM non-essential amino acids. The cells were subcultured by trypsinization [0.05% (wt/vol) trypsin, with 1% EDTA] and plating in growth medium in a humidified 5% CO_2 incubator at 37°C every 2 to 3 days. The cDNA fragments of hARL5 or its mutants were fused in-frame to the C-terminus of GFP by subcloning into the *Eco*RI and *Sal*I sites of pEGFP-C2 (Clontech). Cells were seeded on coverslips 16 hours before transfection with the aid of Lipofectamine (Life Technologies). Freshly prepared solution A (2 μg of plasmid DNA in 50 μl DMEM) and solution B (6 μl Lipofectamine in 50 μl DMEM) were gently mixed for 30 minutes at room temperature, added to 400 μl of DMEM and incubated with cells for 6 hours at 37°C. Additional growth medium with 20% FBS (500 μl) was then added without removing the transfection mixture. Medium was replaced with fresh growth medium the day after transfection and cells were harvested 30 to 36 hours later for analysis.

Indirect immunofluorescence staining

Cells were fixed with 4% paraformaldehyde in PBS-Ca^{2+}-Mg^{2+} (0.6 mM $CaCl_2$ and 0.5 mM $MgCl_2$ in PBS) for 15 minutes, incubated with 0.1% Triton X-100 and 0.05% SDS in PBS-Ca^{2+}-Mg^{2+} for 4 minutes, and in the same buffer containing 0.2% BSA for an additional 15 minutes, followed by incubation with primary antibodies [i.e. affinity-purified anti-hARL5-peptide, mouse anti-p58 (Sigma), mouse anti-β-COP (Sigma), affinity-purified rabbit anti-β-COP peptide (N′-QRKEAADPLASKLNKC-C′), mouse anti-Flag antibody (M2, Sigma), or mouse anti-C23 (nucleolin, Santa Cruz)] in the same solution for 40 minutes. After three washes with PBS-Ca^{2+}-Mg^{2+}, cells were incubated with second antibody, Alexa 594-conjugated anti-rabbit IgG antibody or Alexa 488-conjugated anti-mouse IgG antibody (Molecular Probes), washed three times with PBS-Ca^{2+}-Mg^{2+}, mounted on Mowiol (supplemented with Hoechst 33258), and examined with a Zeiss Axiophot equipped for epifluorescence according to standard procedures (Dascher and Balch, 1994). Primary antibodies, previously depleted of anti-ARL5 activity by incubation with purified recombinant hARL5, were used as control.

Yeast two-hybrid screen and assay

Yeast strains (L40), plasmids (pBTM116 and pVP16), and library for the yeast two-hybrid screen were obtained from H. Shih. The genotype of the *Saccharomyces cerevisiae* reporter strain L40 is *MAT**a** trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ* (Hollenberg et al., 1995). Yeast strains were grown at 30°C in rich medium (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic minimal medium with appropriate supplements. PCR fragment of the ARL5, ARL5(Q80L), ARL5(T35N), ARL5(dMIR), or ARL5(dC) cDNA were generated by using ARL5 and its mutants as template (as described above) and ARL5I and ARL5B as primers (Table 1). Plasmids pLexA-ARL5, pLexA-ARL5(Q79L), pLexA-ARL5(T35N), pLexA-ARL5(dMIR), and pLexA-ARL5(dC), constructed, respectively, by inserting a PCR-generated fragment of the ARL5, ARL5(Q80L), ARL5(T34N), ARL5(dMIR), or ARL5(dC) cDNA into the *Eco*RI site of the pBTM116 plasmid, were used to express the ARL as a fusion protein with the DNA-binding domain of LexA as described previously (Lin et al., 2000). Control plasmids pLexA-ARL1, pLexA-ARL3, pLexA-ARL4, pLexA-ARL4(Q79L), and pLexA-ARL4(T34N) were used and have been described previously (Lin et al., 2000). pACT2-HP1β, -HP1γ, and -HP1α deletion constructs were kindly provided by Pierre Chambon, Anne Dejean, Jacob Seeler and Howard Worman (Seeler et al., 1998; Nielsen et al., 2001).

For two-hybrid screening, the yeast reporter strain L40, which contains the reporter genes *lac*Z and *HIS3* downstream of the binding sequences for LexA, was transformed with pLexA-ARL5-Q80L and a human liver pACT2 cDNA library (Clontech) by the lithium acetate method (Ito et al., 1983), and subsequently treated as described (Hollenberg et al., 1995). Double transformants were plated with synthetic medium lacking histidine, leucine, tryptophan, uracil, and lysine. Plates were incubated at 30°C for three days. His^+ colonies were patched on selective plates and assayed for β-galactosidase activity by a filter assay (Hollenberg et al., 1995). Plasmid DNA was prepared from colonies displaying a $HIS^+/lacZ^+$ phenotype by electrotransformation of HB101 cells and used to re-transform the L40 strain containing the appropriate pLexA-ARL1, pLexA-ARL1(Q71L), pLexA-ARL2, pLexA-ARL3, pLexA-ARL4, pLexA-ARL4(Q79L), pLexA-ARL4(T34N), pLexA-ARL5, pLexA-ARL5(Q80L), pLexA-ARL5(T35N), pLexA-ARL5(dC), pLexA-ARL5(dMIR), and pLexA-lamin, to test for specificity. For assay of β-galactosidase activity, transformants were grown in histidine-containing medium, lysed, and assayed as described (Hollenberg et al., 1995).

In vitro translation and in vitro interaction

In vitro translation reactions were carried out using the rabbit reticulocyte lysate TNT system from Promega (Madison. WI). Briefly 1 μg of DNA was added to a 25-μl TNT reaction containing 12.5 μl of rabbit reticulocyte lysate, 1 μl of TNT reaction buffer, 10 U of RNA polymerase, 0.5 μl of amino acid mix (minus methionine), 20 μCi of [^{35}S] methionine and 10 U of RNasin. After incubation at 30°C for 3 hours, 5 μl of the reaction mix were analyzed by SDS-PAGE and autoradiography. Glutathione-*S*-transferase (GST) fusion proteins, GST-HP1α or GST-importin-α, were synthesized in *E. coli* BL21 by induction with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37°C. Cell pellets were lysed and subsequently sonicated. GST fusion proteins (~10 μg) bound to glutathione Sepharose beads (50 μl) in GST-binding buffer [50 mM KCl, 20 mM HEPES (pH 7.9), 2 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 0.5% nonfat dry milk, and 5 mM dithiothreitol] were mixed with 5 μl of in vitro-translated proteins of interest and incubated at 4°C for 1 hour. Beads were then washed four times with 1 ml of the same buffer. Bound proteins were eluted by boiling in 20 μl of 2× protein sample buffer and separated by SDS-PAGE in 12% gel. The input lane in each experiment contained 10% of the total amount analyzed. GST fusion proteins were stained with Coomassie blue to evaluate equal loading, and bound proteins were visualized after autoradiography.

In vivo interaction

COS-7 cells were grown in DMEM with glutamine penicillin/streptomycin (Gibco) and 10% fetal bovine serum and transfected using Lipofectamine (Life Tech.). Cell transfected with hARL5(WT), (Q80L), (T35N), (dC), or (dMIR) in pEGFP-C2 or with Flag-tagged HP1α in pCMV-tag2 were harvested 48 hours later and proteins detected by western blotting using either a rabbit polyclonal anti-GFP antibody or a mouse monoclonal anti-Flag antibody (M2, Sigma). For immunoprecipitation of the HP1α- hARL5 complex, cells were co-transfected with the appropriate hARL5 construct and HP1α, harvested 48 hours later, washed three times in PBS by centrifugation at 200 ***g*** for 10 minutes at 25°C, dispersed in 300 μl of PBS, and placed on ice. DSP {[dithiobis (succinimidylpropionate)], Pierce} was then added to a final concentration 1 mg/ml. After 1 hour at 0°C, reaction was stopped by addition of 1 M Tris-HCl (pH 7.5) to a final concentration of 20-50 mM, and 15 minutes later cells were harvested, washed twice in PBS and once in binding buffer (TBS: 50 mM Tris-HCl, pH 7, 0.15 M NaCl). Cells were lysed by sonification in 1 ml of binding buffer containing 1 mM phenylmethylsulfonyl fluoride plus other protease inhibitors (leupeptin, aprotinin, chymostatin, antipain, and pepstatin, each 1 μg/ml), and lysates were centrifuged (10,000 ***g***, 15 minutes). As recommended by the manufacturer, 30 μl of M2 anti-Flag affinity gel (Sigma) were added to the supernatant, and the resulting mixture was incubated at 4°C for 3 hour with agitation. After three washes in TBS, beads were dispersed in appropriate amounts of sample buffer for western blotting analysis of bound proteins using anti-GFP polyclonal antibody.

Expression and detection of myristoylated ARL5

To produce myristoylated proteins, BL21(DE3) competent bacteria were co-transformed with pT7-ARL5 and pACYC177/ET3d/yNMT, which encodes yeast (*S. cerevisiae*) *N*-myristoyltransferase (Haun et al., 1993), and selected for both ampicillin and kanamycin resistance. [9,10(n)-^{3}H]myristic acid (1 mCi/ml in ethanol; Amersham) was added to a final concentration of 30 μCi/ml and the cultures were further treated as previously described (Huang et al., 1999).

Metabolic labeling and immunoprecipitation

COS-7 cells were cultured in Dulbecco's modified Eagle's medium(DMEM) containing 10% fetal bovine serum and glutamine at 37°C in a humidified 5% CO_2 atmosphere. Cells in 6-well plates (1×10^5 cells/well) were grown for 16 hours before transfection with 1 μg of pcDNA3.1A containing hARL5(WT) or hARL5(G2A) mutant cDNA and 7 μl of LipofectAMINE reagent (Life Technologies) in 1 ml of serum-free DMEM. After incubation for 5 hours, 1 ml of DMEM containing 20% fetal bovine serum was added to each well and 36 hours after transfection, cells were incubated for 12 hours with 1 ml of Dulbecco's modified Eagle's medium containing 2% fetal bovine serum and 150 μCi [9,10-^{3}H]myristic acid (Amersham Pharmacia Biotech.). The [9,10-^{3}H]myristic acid had been dried under N_2 and dissolved in DMSO. Final concentration of DMSO in the medium was 0.1%. Cells were washed with PBS and lysed in 2 ml of radioimmunoprecipitation buffer (50 mM Tris-HCl(pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxy cholate, 0.1% SDS, proteinase inhibitors) for 40 minutes at 4°C. Cell lysates were centrifuged (5 minutes, 15,000 ***g***) at room temperature in a microcentrifuge, and supernatants were collected for

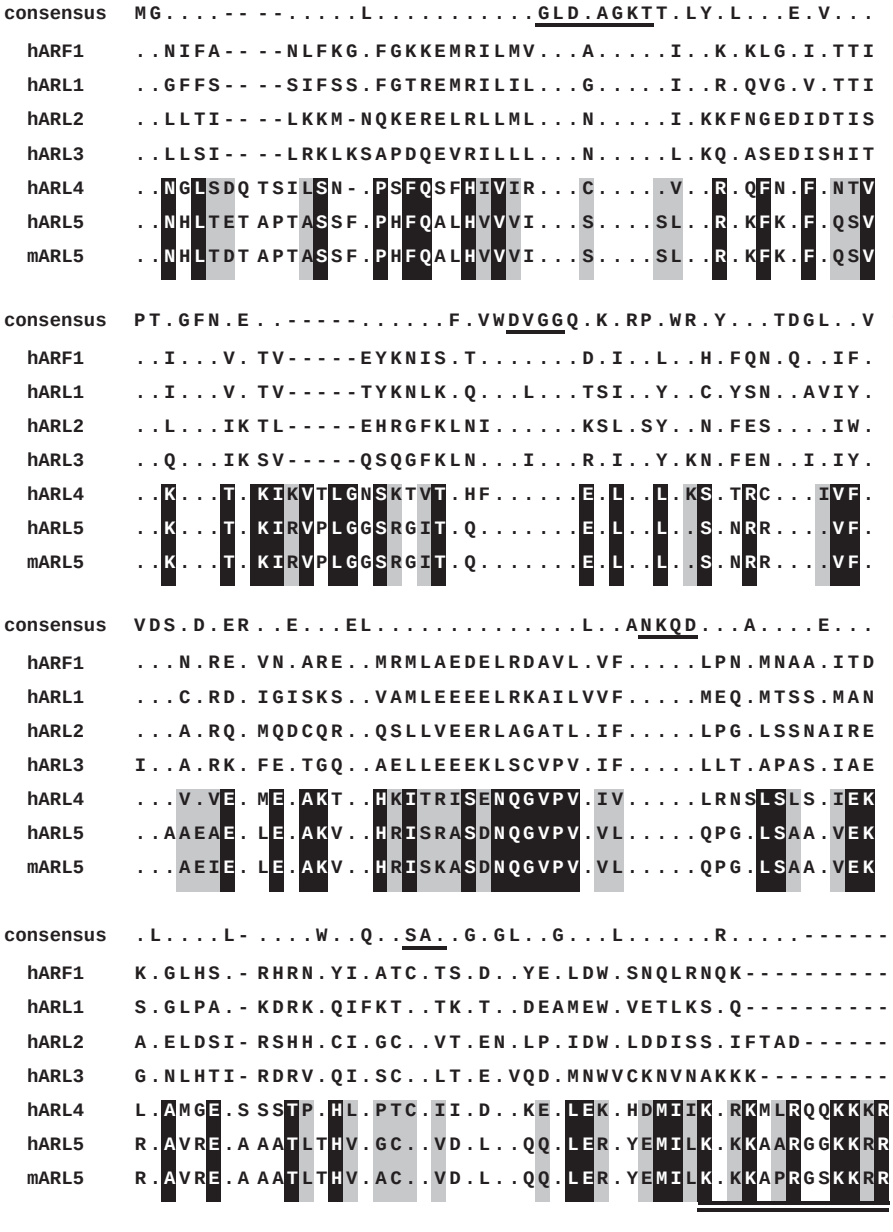

Fig. 1. Alignment of deduced amino acid sequences of ARL5, ARLs and ARF1. Sources of sequences are: hARF1 (Bobak et al., 1989); hARL1 (Zhang et al., 1995); hARL2 (Clark et al., 1993); hARL3 (Cavenagh et al., 1994); hARL4 (Lin et al., 2000); hARL5 [this study (Smith et al., 1995)]; mARL5 (this study). Amino acids identical in at least five of the seven sequences compose the consensus sequence shown at the top. Underlines indicate consensus sequences for GTP binding and hydrolysis. Double underline indicates consensus sequences for nuclear localization signal. Black boxes indicate amino acids identical in hARL4, hARL5 and mARL5 sequences. Gray boxes indicate amino acids similar in hARL4, hARL5 and mARL5 sequences.

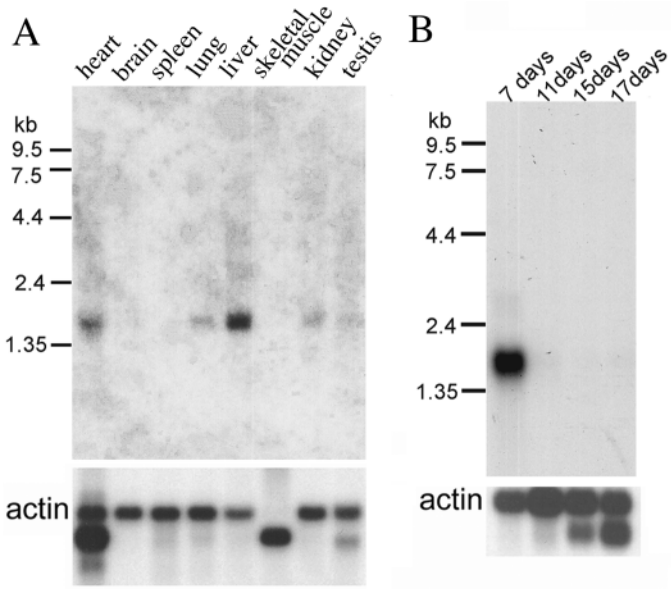

Fig. 2. ARL5 mRNA in mouse tissue and embryos. Blots containing poly $(A)^+$ RNA from adult mouse tissues (A) or whole embryos at different developmental stages (B) were hybridized with a random-primed, ^{32}P-labeled mARL5 cDNA probe. Hybridization with a β-actin probe was a control for sample loading.

immunoprecipitation. Following clearance with protein A-Sepharose, to each ml of supernatants, 10 μl of anti-myc monoclonal antibody (9E10) was added. After rocking at 4°C for 6 hours, 30 μl of protein A-Sepharose was added, and the samples were tumbled overnight at 4°C. Beads were pelleted by centrifugation (30 seconds, 600 ***g***) and washed five times with immunoprecipitation buffer. Bound proteins were eluted in 20 μl of 2× SDS sample buffer and separated by SDS-PAGE in 12% gel. Gels were fixed, dried, and exposed to Hyperfilm for 21 days at –80°C using.

CTA (cholera toxin A subunit)-catalyzed ADP-ribosylation and nucleotide binding assay

Samples (5 μg) of purified His-tagged hARL5 or yARF1 were tested for their ability to stimulate cholera toxin-catalyzed auto-ADP-ribosylation (Huang et al., 1999). Binding of GTPγS to purified recombinant hARL5 was determined by a filtration method (Northup et al., 1983) with minor modification as previously described (Lee et al., 1997).

Results

Analyses of protein sequences of ARL5

Human and mouse ARL5 (hARL5 and mARL5) were identified as products of PCR amplification using degenerate probes derived from conserved sequences in members of ARL family (see Materials and Methods). Predicted amino acid sequences (201 residues) of human and mouse ARL5 are 96% identical, differing only at positions 7, 103, 106, 121, 167, 194

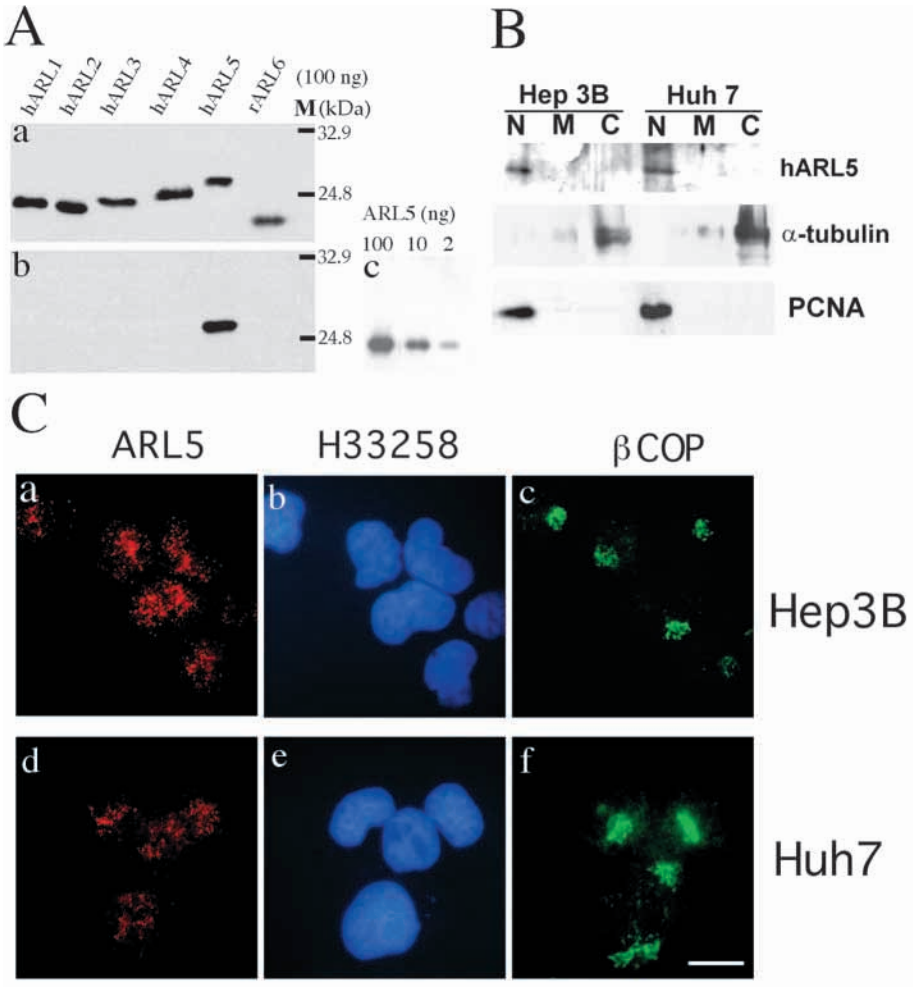

Fig. 3. Immunolocalization of endogenous hARL5 in hepatoma Hep3B and Huh7 cells. (A) Specificity of antibody against ARL5. ~100 ng of the indicated purified recombinant His-tagged ARL (a,b) and the indicated amounts of purified His-tagged hARL5 (c) were subjected to SDS-PAGE in 12.5% gels. Positions of protein standards (32.9 and 24.8 kDa) are on the left. Proteins were transferred to nitrocellulose and reacted with (a) anti-His-tag or (b,c) anti-ARL5 antibodies, followed by detection using the ECL system (A,B). (B) Subcellular distribution of hARL5. Nuclear (N), membrane (M) and cytosolic (C) fractions of Hepatoma Hep3B or Huh7 cells were prepared as described in Materials and Methods. Equivalent amounts (from total homogenate) of each fraction were analyzed by electrophoresis and immunoblotting using specific antibodies against ARL5, α-tubulin (cytosolic marker), or PCNA (nuclear marker). (C) Immunolocalization of ARL5. Hepatoma Hep3B and Huh7 cells on glass coverslips, treated as described in Materials and Methods, were incubated with affinity-purified anti-hARL5-peptide (a,d) or βCOP antibodies (c,f). Coverslips were mounted on Mowiol (supplemented with Hoechst 33258; b,e) and inspected with a Zeiss Axiophot equipped for epifluorescence. Bar, 10 μm.

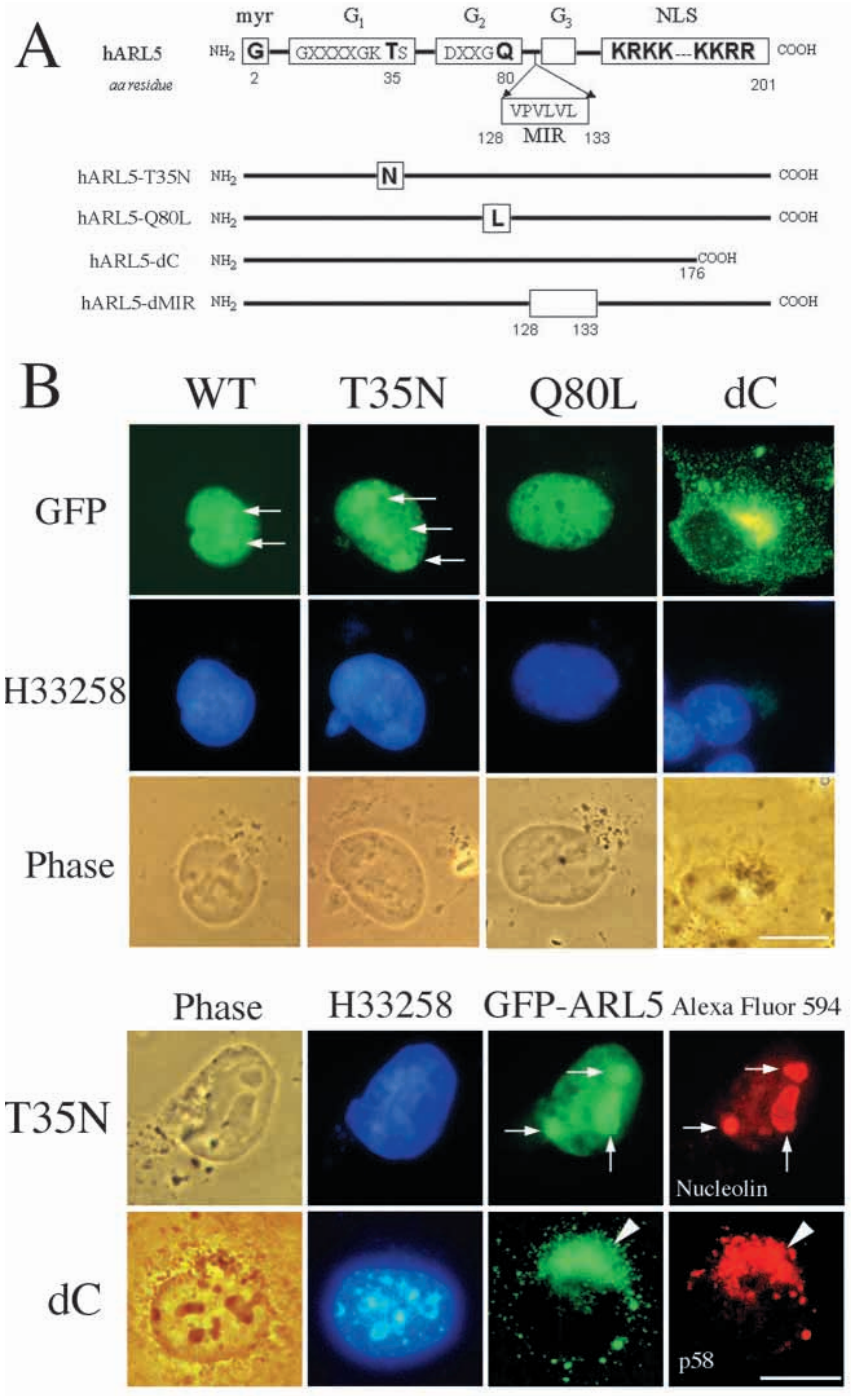

Fig. 4. Transient expression of hARL5 and its mutants in COS-7 cells. (A) Diagrams of wild-type (WT) hARL5 and four hARL5 mutants with amino acid numbers below. Residues 188 to 201 contain the bipartite NLS of ARL5. ARL(dC) lacks 24 amino acids (177 to 201) at the C-terminus. Positions of the mutations Q80L and T35N, and MIR deletion (128-133) are also indicated. (B) COS-7 cells transfected with GFP fusion constructs of hARL5(WT), hARL5(Q80L), hARL5(T35N), or hARL5(dC) were grown on glass coverslips, fixed with formaldehyde, incubated with mouse anti-nucleolin (nucleolar marker), and detected with Alexa-488-conjugated anti-mouse IgG antibody. Coverslips were mounted on Mowiol (supplemented with Hoescht 33258) and inspected with a Zeiss Axiophot equipped for epifluorescence. Phase-contrast microscopy and GFP-hARL5 fluorescence (green) are shown. In the bottom two rows, arrows indicate nucleolins, and arrowheads indicate Golgi marker p58. Bar, 10 μm

and 197. Sequences of the ARL5, other ARL proteins and ARF1 are aligned in Fig. 1. The deduced sequences of hARL5 and hARL4 are 60% identical and 79% similar, although ARL5 is only 29-43% identical to other ARLs and ARFs. Like other ARL proteins, ARL5 has a glycine at position 2, the site of N-myristoylation in ARF/ARL proteins. ARL5 lacks cysteine residues near the carboxyl terminus, which are sites of isoprenylation in non-ARF members of the Ras superfamily. ARL5 also contains the ARF family consensus sequences for guanine nucleotide binding (DVGG, NKQD and CAT) and GTP hydrolysis (GXXXXGKT) (reviewed by Moss and Vaughan, 1995). Moreover, ARL5, similar to ARL4, contains an additional C-terminal putative bipartite NLS, that is, K^{189}RKKAARGGKKRR^{201}.

Developmentally regulated expression of mARL5

On Northern blot analysis, a ~1.4-kb transcript of mARL5 was more abundant in liver than in other adult mouse tissues (Fig. 2A). In mouse embryos, the level of mARL5 mRNA was high on embryonic day 7 and hardly detected by day 11 (Fig. 2B). We have tried, thus far without success, to localize mARL5 RNA in mouse embryos by in situ hybridization. This is reminiscent of the low level of ARL5 mRNA in the day-11 embryo, and makes it difficult to establish tissue distribution of mARL5 before day-8 mouse embryo.

Subcellular localization of ARL5

To identify the intracellular location of ARL5 protein, we prepared ARL5-specific antibodies against a unique peptide sequence (residues 2-18) of human ARL5. The affinity-purified antibodies are sensitive and specific for detection of both human and mouse ARL5 proteins. Immunoblotting with this antiserum detected ARL5 in low nanogram amounts, while no reaction was detected with 100 ng of other recombinant ARLs (Fig. 3A).

To assess the subcellular distribution of ARL5 in human hepatoma Hep3B cells, homogenates were fractionated by differential centrifugation. Nuclear (N), membrane (M), and cytosol (C) fractions were separated and hARL5, PCNA (proliferating cell nuclear antigen), and tubulin (cytoplasmic marker) in subcellular fractions were identified by western blot analysis (Fig. 3B). Endogenous hARL5 was detected in the nuclear fraction (Fig. 3B). Immuno-reactivity with hARL5 was abolished by prior incubation of the antiserum with recombinant hARL5 (data not shown). By

immunofluorescence microscopy, endogenous hARL5 in Hep3B and Huh7 cells was distributed mainly over the nucleus (Fig. 3C). No immunoreactivity was detected with preimmune serum or after incubation of antibody ARL5-N with purified recombinant hARL5 (not shown). Nuclei were stained with the DNA-binding dye H33258 (Fig. 3Cb,e) and Golgi with anti-β-COP antibodies (Fig. 3Cc,f).

Nucleolar localization of ARL5-T35N mutant

To determine whether the subcellular localization of hARL5, like that of hARL4, was dependent on GTP or GDP binding (Lin et al., 2000), COS-7 cells transiently expressing GFP-tagged hARL5, hARL5(Q80L) (predicted to be GTP-bound), hARL5(T35N) (predicted to be GDP-bound), or hARL5(dC) (lacking the NLS) were inspected by fluorescence microscopy (Fig. 4). hARL5 appeared to be located in nuclei and partially in nucleoli. hARL5(T35N), but not hARL5(Q80L), appeared to be more concentrated in nucleoli (Fig. 4, arrows). Co-localization of hARL5(T35N) with nucleolin, a marker for nucleoli, confirmed its nucleolar localization. hARL5(dC), not seen in nuclei, distributed, in part, in a punctate pattern in the cytoplasmic region and, in part, co-localized with the Golgi marker p58 (Fig. 4, arrowheads). When subcellular distribution of transiently expressed hARL5 and its mutants in homogenates of COS-7 cells was examined, hARL5, hARL5(Q80L), and hARL5(T35N) were detected mainly in the nuclear fraction, with very little in the membrane fraction

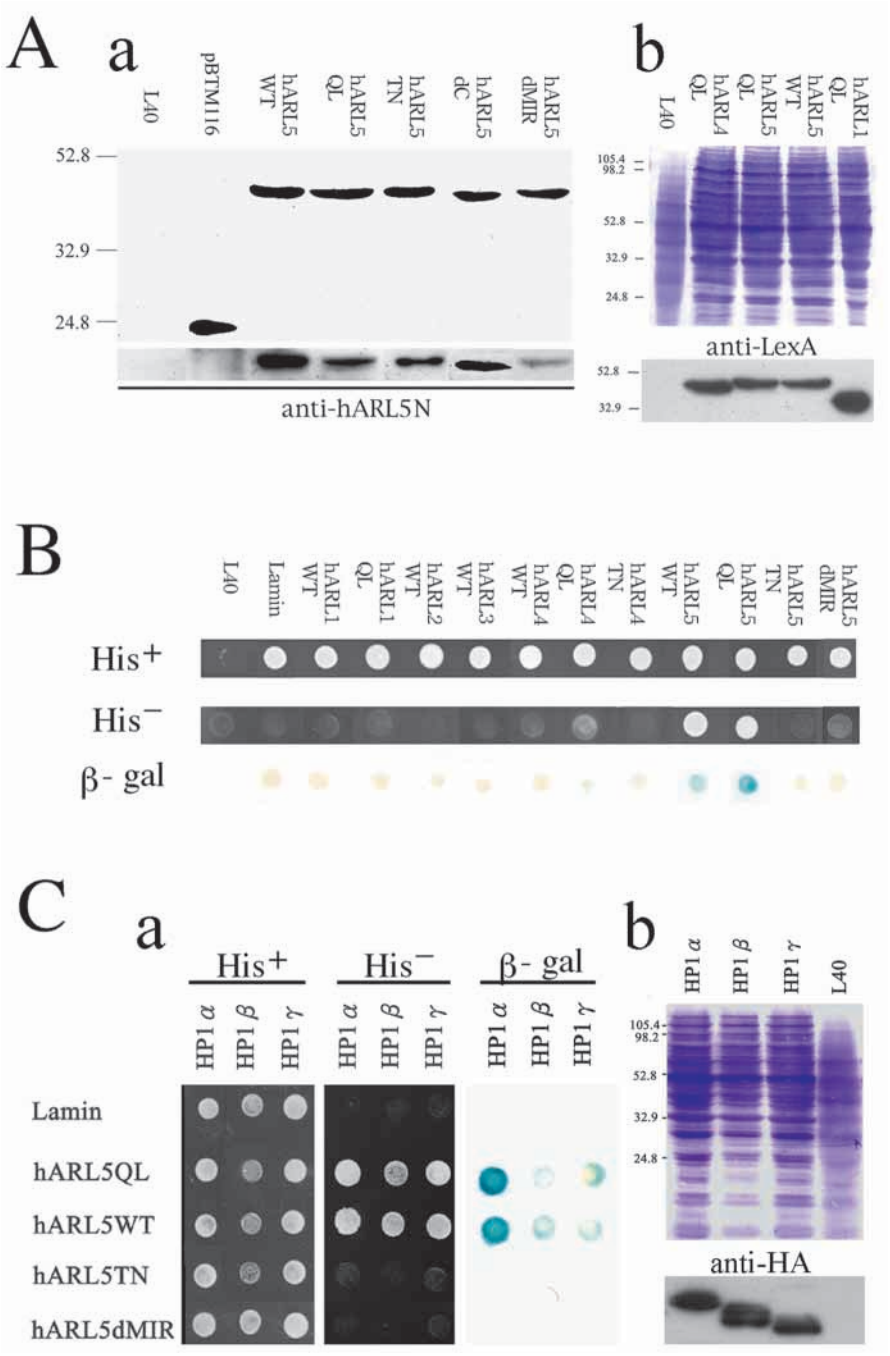

Fig. 5. Interaction of hARL5 with HP1 in the two-hybrid system. (A) Expression of LexA-ARL fusion proteins. Yeast reporter strain L40 was transformed with the indicated LexA construct, pBTM116 (LexA only), or pLexA-lamin. Samples (~20 μg) of cell lysates were subjected to SDS-PAGE in a 10% gel, then stained with Coomassie blue (b, upper panel). Proteins were transferred to nitrocellulose and reacted with anti-LexA antibodies (a, upper panel; b, lower panel), or anti-ARL5 antibodies (a, lower panel) as indicated, followed by detection using the ECL system.
(B) Interaction of hARL5 and mutants with HP1α in the two-hybrid system. Yeast reporter strain L40 co-transformed with pACT2-HP1α and the indicated pLexA-ARL construct or pLexA-lamin were plated on synthetic histidine-containing medium lacking leucine, tryptophan, uracil and lysine (His^+ plate, upper panel). Colonies from His+ plates were assayed for β-galactosidase activity by a filter assay to test for specificity (lower panel). Colonies from His^+ plates were also patched on His^- selective plates lacking histidine, leucine, tryptophan, uracil, and lysine (His^- plate, middle panel). (C) Interaction of hARL5 and mutants with HP1α, HP1β, and HP1γ in the two-hybrid system. Yeast reporter strain L40 co-transformed with pACT2-HP1α, -HP1β, or -HP1γ, and the indicated pLexA-ARL5 construct or pLexA-lamin were plated and assayed as described above (a). Proteins were transferred to nitrocellulose and reacted with anti-HA antibodies (b, lower panel), followed by detection using the ECL system. Samples (~20 μg) of transformed yeast lysates were subjected to SDS-PAGE in a 10% gel, then stained with Coomassie blue (upper panel b). Proteins were transferred to nitrocellulose and reacted with anti-HA antibodies (b, lower panel), followed by detection using the ECL system. Positions of protein standards (kDa) are on the left.

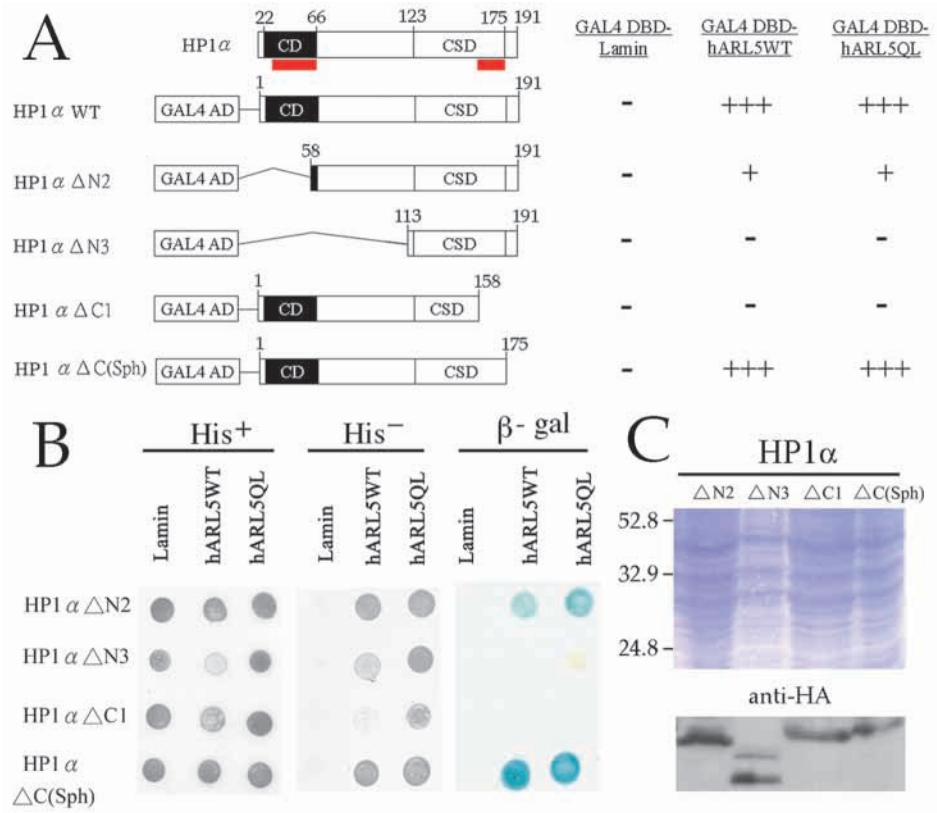

Fig. 6. Interaction of hARL5 and mutants with four HP1α deletion derivatives in the two-hybrid system. The diagram of HP1α and its deletion derivatives was adapted from Seeler et al., 1998 (Seeler et al., 1998) (A, left). The adapted figure was reproduced with permission from National Academy of Sciences, USA. Yeast reporter strain L40 co-transformed with pACT2-HP1α deletion derivatives and the indicated pLexA-ARL5 construct or pLexA-lamin were plated and assayed as described above (B). Panel A (right) shows the summary of the interaction result. Samples (~20 μg) of transformed yeast lysates were subjected to SDS-PAGE in a 10% gel (C, upper panel). Positions of protein standards (kDa) are on the left. Proteins were transferred to nitrocellulose and reacted with anti-HA antibodies (C, lower panel), followed by detection using the ECL system. Red underlines (A) indicate regions of HP1α that interact with ARL5.

(data not shown). hARL5(dC), however, appeared mainly in the membrane fraction, confirming the results of fluorescence microscopy.

Identification of HP1α as a binding partner of ARL5

To identify molecules that might act as down-stream effectors of hARL5, we used plasmid pLexA-hARL5(Q80L) to express the putatively constitutively active mutant of hARL5 (hARL5(Q80L)) as bait in a yeast two-hybrid screen of a human liver cDNA library (Hollenberg et al., 1995). The bait caused no intrinsic transcriptional activation of the reporters. Plasmids associated with β-galactosidase production were identified from a screen of approximately 4×10^6 colonies. The DNA sequence of each library insert was determined and five different inserts were chosen for further analysis. DNA sequencing and database searches revealed that nucleotide sequences of three clones encoded human HP1α (accession number L07515), two were full-length and one encoded amino acids 26-191. Thus, the interaction of HP1α with ARL5 appeared not to involve its N-terminus. Recently, a MIR motif, VPVVVL, has been proposed to be a consensus sequence for HP1α binding (Murzina et al., 1999; Smothers and Henikoff, 2000). Because, a similar sequence, VPVLVL (aa 128-133), is present in ARL5 (Fig. 4A), we constructed a mutant lacking this motif (hARL5(dMIR)) to assess its interaction with HP1α. We also used wild type hARL5 and hARL5(T35N) to test whether the interaction with HP1 is nucleotide-dependent. The LexA-ARL5 fusion proteins were expressed in yeast and detected by antibodies against LexA or ARL5 (Fig. 5Aa). In the yeast two-hybrid assay, transformants containing interacting proteins that transactivate two reporter genes, *HIS3* and *LacZ*, exhibit β-galactosidase activity and can grow on minimal medium lacking histidine. As illustrated in Fig. 5B, LexA-hARL5 and LexA-hARL5(Q80L), but not LexA-hARL5(T35N), LexA-hARL5(dMIR), LexA-hARL1, LexA-hARL3, LexA-hARL4, or LexA-hARL4(T34N), interacted with the Gal4AD-HP1α fusion protein and activated the reporter genes. Interaction of LexA-hARL4(Q79L) with the Gal4AD-HP1α fusion protein was much less than that of LexA-hARL5(Q80L). The LexA-hARL4(Q79L) and LexA-hARL5(Q80L) were present in relatively equal amounts in each of the transformed yeast (Fig. 5Ab). At least three HP1 family proteins (HP1α, HP1β, and HP1γ) have been characterized (reviewed by Eissenberg and Elgin, 2000). Interaction of Gal4AD-HP1β and Gal4AD-HP1γ with the LexA-hARL5(WT) and LexA-hARL5(Q80L) fusion protein was much less than the HP1α interaction (Fig. 5Ca). The Gal4AD-HP1α, Gal4AD-HP1β and Gal4AD-HP1γ were also found in relatively equal amounts in the transformed yeast (Fig. 5Cb). To identify the ARL5-interacting domain of HP1α, in the yeast two-hybrid assay (Fig. 6A), we used four deletion derivatives, which were expressed in relatively equal amounts (Fig. 6C). Two regions (residues 1-58 and 158-175) of HP1α appeared to be important for ARL5 interaction (Fig. 6A,B).

To confirm that the interactions between hARL5 and HP1α were direct, hARL5 and its mutants were synthesized in an in vitro translation system and incubated with GST-HP1α immobilized on glutathione Sepharose. As shown in Fig. 7A, recombinant GST-HP1α, but not GST, adsorbed significant amounts of hARL5 and hARL5(Q80L), whereas no binding of hARL5(T35N) or hARL5(dMIR) was detected.

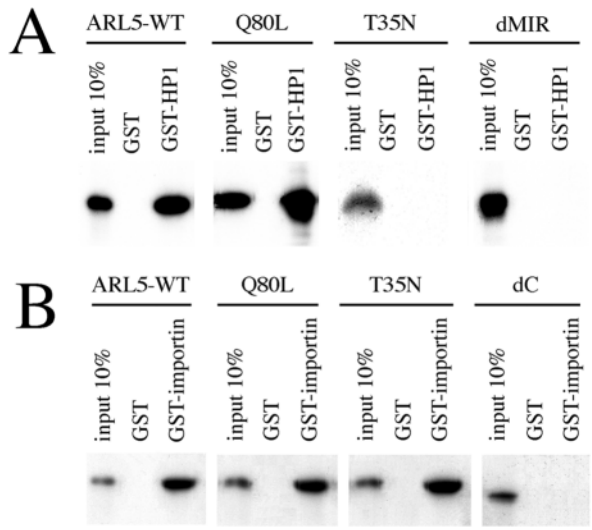

Fig. 7. In vitro interaction of hARL5 with HP1α and importin-α. (A) In vitro interaction of GST-HP1α with hARL5 constructs. hARL5, hARL5(Q80L), hARL5(T35N), hARL5(dC), or hARL5(dMIR) were synthesized by in vitro translation. Samples (5 μl) were mixed with 10 μg of GST or GST-HP1α immobilized on glutathione-Sepharose beads and incubated at 4°C for 1 hour before beads were washed four times with 1 ml of the same buffer. Bound proteins were eluted by boiling in 20 μl of 2× protein sample buffer and separated by SDS-PAGE in 12% gel. Input lane contained 10% of the amount added to beads. Proteins were stained with Coomassie Blue to ensure equal loading, and the bound proteins were visualized by autoradiography. (B) In vitro interaction of GST-importin-α with hARL5 constructs. 10 μg of GST or GST-importin-α immobilized on glutathione-Sepharose beads were mixed with 5 μl of in vitro translated proteins of interest as described as above.

Interaction of importin-α with ARL5 C-terminal nuclear localization signal NLS

Because hARL5, like hARL4, contains a putative bipartite NLS (K^{189}RKKAARGGKKRR^{201}) at its C-terminus (Fig. 4A), we used a hARL5 mutant lacking this sequence, (hARL5(dC)), to test its interaction with importin-α, (karyopherin alpha 2; accession number NM_002266) (Cuomo et al., 1994; Weis et al., 1995), which had also been isolated in the two-hybrid screening. LexA-hARL5, LexA-hARL5(Q80L), and LexA-hARL5(T35N), but not LexA-hARL5(dC), LexA-hARL1, or LexA-hARL3, interacted with the Gal4AD-importin-α fusion protein and activated the reporter genes (data not shown). An in vitro GST pull-down assay confirmed that the interactions between ARL5 and importin-α are direct. hARL5 and its mutants, produced by in vitro translation, were incubated with immobilized GST-importin-α in vitro. As shown in Fig. 7B, recombinant GST- importin-α, but not GST, adsorbed significant amounts of ARL5, ARL5(Q80L), and ARL5(T35N), whereas no binding of ARL5(dC) was detected. Binding of ARL5 and the two mutants (expected to exist largely in GTP- or GDP-bound forms) was not grossly different.

Intracellular localization and interaction of hARL5 and its mutants with HP1α

To assess the interaction of hARL5 and HP1α in vivo, we compared the subcellular localization of hARL5 and its mutants with that of HP1α. COS-7 cells transiently co-expressing Flag-tagged HP1α and GFP-tagged hARL5, hARL5(Q80L), hARL5(T35N), ARL5(dMIR), or ARL5(Q80L/dMIR) were inspected by fluorescence microscopy (Fig. 8). hARL5(Q80L), and to a lesser extent hARL5, was in part co-localized with overexpressed HP1α (Fig. 8), and this was apparently abolished by deletion of MIR motif. ARL5(dMIR) and ARL5(Q80L/dMIR) apparently, in part, localized to nucleoli (Fig. 8q,t,u,x). Distribution of ARL5 and its mutants were similar to those shown in Fig. 4. hARL5(dC) was, in part, distributed in a punctate pattern in the cytoplasmic region and, in part, co-localized with the Golgi marker (data not shown). After subcellular fractionation, transiently expressed ARL5(dMIR) was detected mainly in the nuclear fraction with very little in the membrane fraction, confirming the results of fluorescence microscopy (data not shown).

For immunoprecipitation of the HP1α-hARL5 complex, cells were co-transfected with the appropriate hARL5 construct and HP1α. As shown in Fig. 9, recombinant GST-HP1α co-immunoprecipitated with hARL5 and hARL5(Q80L), whereas no association with hARL5(T35N), or hARL5(dMIR) was detected. These data extend the earlier findings and confirm the importance of the GTP-bound state of the hARL5(Q80L) and of the MIR motif for formation of a hARL5-HP1α complex in cells.

Biochemical properties of ARL5 protein

The hARL5 fusion protein, like those of hARL2 and hARL3, failed to stimulate auto-ADP-ribosylation of the cholera toxin A1 protein (data not shown). hARL5 did bind GTPγS in a concentration-dependent manner that reached a steady state within 60 minutes at 30°C. Phospholipids that increased GTPγS binding by hARF1 markedly decreased binding by hARL5 as they did binding by ARL4 (data not shown).

To determine whether hARL5 could be myristoylated, hARL5, hARL1, and yARL3 were co-expressed in *E. coli* with yeast N-myristoyltransferase. hARL5 and hARL1 [as reported previously (Lee et al., 1997)] were myristoylated (Fig. 10A). yARL3, previously shown not to be myristoylated (Huang et al., 1999), served as a negative control. Because of the low affinity of our anti-ARL5N peptide antibody, we have failed to isolate endogenous ARL5 to assess its myristoylation in human cells. We did test whether overexpressed ARL5 could be myristoylated. COS-7 cells were transfected with C-terminal Myc-tagged ARL5(WT) and ARL5(G2A), in which Gly at position two was replaced by Ala. Cells were incubated with [^{3}H]myristic acid. Immunoprecipitated ARL5(WT), but not ARL5(G2A), was [^{3}H]-myristoylated (Fig. 10B). Although two forms of ARL5(WT) and ARL5(G2A) with different mobilities on SDS-PAGE were immunoprecipititated by the anti-Myc antibody, only the upper band reacted with the anti-hARL5N peptide antibody. We believe that the lower bands of both ARL5(WT) and ARL5(G2A) (Fig. 10B, asterisk) were degraded at the N-terminus, and react, therefore, with anti-Myc, but not anti-hARL5N, antibodies. It appears that the biological function of ARL5, like those of ARL1, ARL4, the ARFs, and other proteins, may be influenced by myristoylation.

Discussion

We have characterized the expression, subcellular localization, and certain biochemical properties of a highly conserved small GTPase, ARL5. There are at least three distinct classes of ARLs (Hong et al., 1998). ARL1 (Class I) appears to be different from ARL2 and ARL3 (Class II) and ARL4 and ARL5 (Class III). Deduced amino acid sequences of ARL4 and ARL5 are 60% identical and 79% similar. Our data clearly indicate that embryonic expression of mARL5 mRNA, like that of mARL4, is developmentally regulated. By indirect immunofluorescence and biochemical techniques, we show that localization of hARL5 in nuclei and nucleoli, similar to that of hARL4, is influenced by nucleotide binding. Unlike mARL4, which is abundant in testis, mARL5 is most abundant in liver (Lin et al., 2000). ARL5 and ARL4 differ from other ARLs in the presence of an additional C-terminal putative bipartite NLS, that is, $K^{189}RKKAARGGKKRR^{201}$ (Fig. 1). The C-terminal region of importin-α recognizes the NLS (Moroianu et al., 1996). Interaction of hARL5 (like that of ARL4) with importin-α, apparently, is not GTP-dependent and does require the C-terminal NLS. Joost and colleagues (Jacobs et al., 1999) showed that the basic C-terminus (20 amino acids) of these ARLs, when fused to the C-terminus of GFP, targets the constructs to the nucleus of transfected cells.

Although the role of the nucleolus as a factory for assembling ribosomal subunits is well established, one of the more interesting findings is the presence thereof a variety of macromolecules that have no apparent ribosomal function (reviewed by Garcia and Pillus, 1999; Olson et al., 2000). The nucleolus seems to have roles also in assembling ribonucleoprotein (RNP), modifying small RNAs, controlling aging, sequestering regulatory molecules, and nuclear export. In these events, the nucleolus serves as a site for both recruitment and exclusion of regulatory complexes. Recently, several cell-cycle regulators whose activity is controlled by sequestration in the nucleolus have been identified (reviewed by Visintin and Amon, 2000). To our knowledge, ARL5 and ARL4 may be the first subfamily of small GTPases reported to be localized in nucleoli in a manner dependent on GDP binding. Studies of nuclear architecture reveal that the dynamic properties of nuclear proteins are critical for their function (reviewed by Misteli, 2001). The mobility of proteins facilitates their availability throughout the nucleus, and their dynamic interplay generates an ever-changing, but overall stable, architectural framework, within which nuclear processes take place. Overall, nuclear morphology is

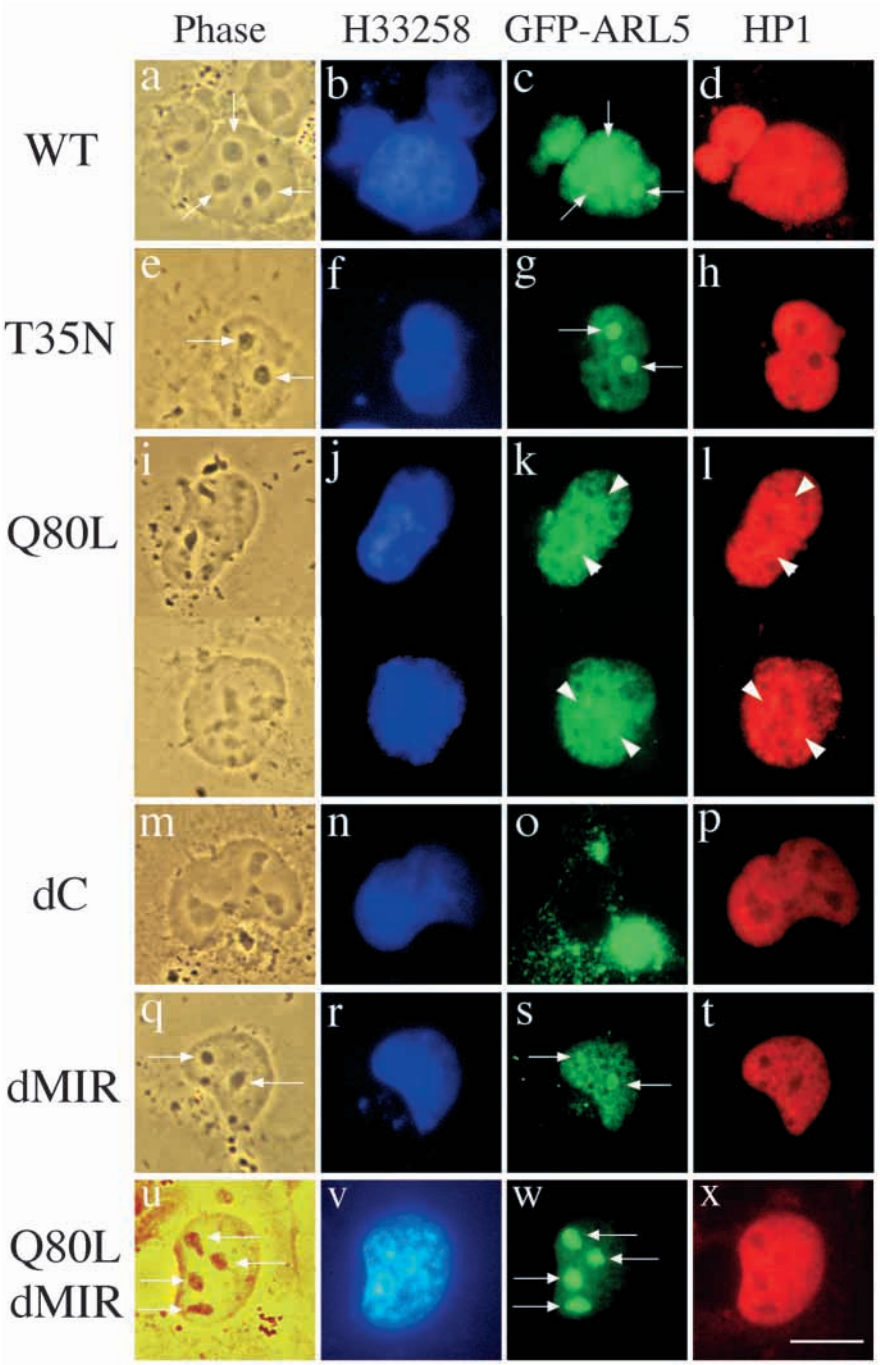

Fig. 8. Transient expression of hARL5 or its mutants and HP1α in COS-7 cells. COS-7 cells co-transfected with Flag-tagged HP1-α and GFP-tagged constructs of hARL5, hARL5(Q80L), hARL5(T35N), hARL5(dC), hARL5(dMIR), or hARL5(Q80LdMIR) were grown on glass coverslips, fixed with formaldehyde, and incubated with mouse anti-Flag (d,h,l,p,t,x) or mouse anti-nucleolin (nucleolar marker) antibodies. Coverslips were mounted on Mowiol (supplemented with Hoechst 33258; b,f,j,n,r,v) and inspected with a Zeiss Axiophot equipped for epifluorescence. Phase-contrast microscopy (a,e,i,m, q,u) and GFP-hARL5 fluorescence (c,g,k,o,s,w; green) are shown. Nucleolins are indicated by arrows. Co-localization of HP1-α and hARL5(Q80L) is indicated by arrowheads. Bar, 10 μm.

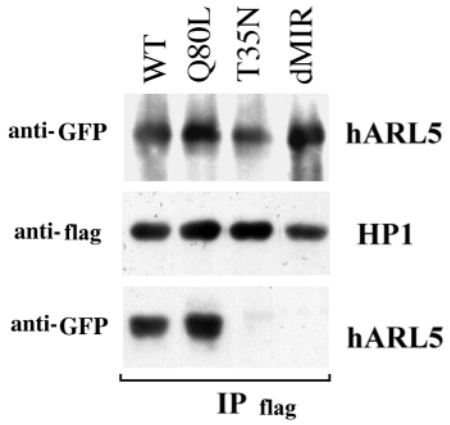

Fig. 9. In vivo interaction of hARL5 with HP1α. For immunoprecipitation of the HP1α- hARL5 complex, COS-7 cells were co-transfected with an hARL5 construct and HP1α. After 48 hours, cells were harvested, washed three times in PBS, suspended in PBS, and placed on ice. DSP was then added. After 1 hour at 0°C, the reaction was stopped. The cells were harvested, washed and lysed. After centrifugation (10,000 ***g***, 15 minutes) of lysates, M2 anti-Flag affinity gel was added to the supernatants, followed by incubation at 4°C for 3 hours with agitation. Beads were then washed three times with TBS and dispersed in appropriate amounts of sample buffer. Eluted proteins were subjected to western blotting analysis using anti-GFP polyclonal antibody (lower panel). Samples of total cell lysates were also subjected to western blotting analysis using anti-Flag and anti-ARL5N antibodies (upper two panels).

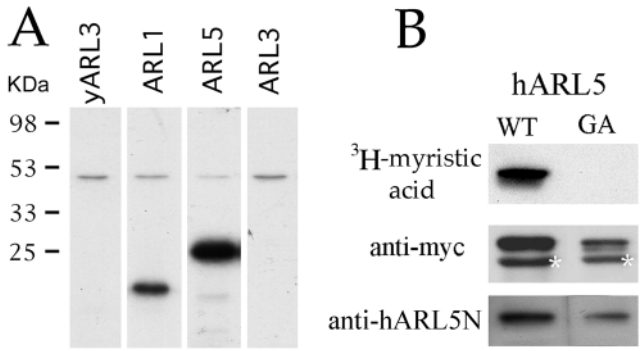

Fig. 10. Myristoylation of hARL5. (A) Recombinant hARL5, hARL1, and yARL3 were synthesized in *E. coli* co-expressing yeast *N*-myristoyltransferase that were grown in medium containing (^{3}H)-myristic acid. Samples of bacterial proteins (~20 μg) were subjected to SDS-PAGE in a 15% gel, which was fixed in 10% acetic acid and 45% methanol for 30 minutes, incubated in Amplify (Amersham) for 20 minutes, dried, and exposed to Hyper-film (Amersham) for 41 hours at –80°C. (B) COS-7 cells were transfected with pcDNA3.1A C-terminal Myc-tagged ARL5(WT) or ARL5(G2A) and metabolically labeled with [^{3}H]myristic acid before immunoprecipitation of cell extracts with anti-Myc antibody. Beads were dispersed in 20 μl of 2× SDS sample buffer and extracted proteins analyzed by SDS-PAGE in 12% gel. Gels were fixed, dried and exposed to Hyperfilm for 21 days at –80°C (upper panel). Part of each immunoprecipitated sample was subjected to western blotting analysis using anti-Myc (middle panel) and anti-ARL5N antibodies (lower panels). Asterisks indicate the lower bands of both ARL5(WT) and ARL5(G2A).

determined by the functional interactions of nuclear components. The dynamic properties of nuclear proteins are consistent with a central role for stochastic mechanisms in gene expression and nuclear architecture. Although the physiological function of ARL5 and ARL4 in nuclei and nucleoli is not understood, it will be important to determine whether ARL5 and ARL4 participate in the regulation of nuclear protein dynamics via their nucleolar sequestration.

Of five proteins that interacted with hARL5 in the yeast two-hybrid screening, one that interacted with ARL5(Q80L) and ARL5(WT), but not ARL5(T35N) or ARL5(dMIR), in the two-hybrid system is a known HP1α. HP1α proteins are believed to represent important structural components of heterochromatin, consistent with their involvement in position-effect variegation (reviewed by Singh, 1994; Elgin, 1996), as well in proper functioning of the centromere during mitosis (Kellum and Alberts, 1995). HP1 proteins are phosphorylated in vivo and this phosphorylation may be important mechanism for regulating their multimerization and/or other interactions. HP1α, HP1β, and HP1γ share two highly conserved globular domains, an N-terminal chromo domain (CD) and a C-terminal chromo shadow domain (CSD), which span residues 22-66 and 123-175 of HP1α, respectively (Eissenberg and Elgin, 2000). The CD and CSD of HP1 family are connected by a poorly conserved hinge region (H). Several cellular proteins have been reported to interact directly with HP1 proteins through the CSD; these include the lamin B receptor (Ye et al., 1997), the transcriptional cofactors TIF1 β and other chromosomal regulators (Le Douarin et al., 1996), the chromatin assembly factor 1 (CAF-1) subunit p150 (Murzina et al., 1999), as well as PML/SP100 nuclear bodies (Seeler et al., 1998). Both CSD and CD domains of HP1 are required for interaction with ORC (Pak et al., 1997). HP1α, via its CD, also binds to histone H3, suggesting a role for histone H3 in anchoring CD-containing proteins to the chromatin fiber (Nielsen et al., 2001). Interactions of HP1α with SWI/SNF complex member BRG-1 and with a RAD-54- like protein called HP-BP-38 also have been reported (Le Douarin et al., 1996). Although the precise functional consequences of these interactions, or of the interaction of ARL5 with HP1α, remain to be determined, these data suggest that HP1α proteins might represent important targets for cellular regulators of DNA transcription, replication, or repair.

Several proteins, such as the p150 subunits of CAF-1, TIF1α, and TIFβ, bound to HP1α directly through an amino-terminal sequence, termed MIR for MOD1 (mouse HP1α)-interaction region (Murzina et al., 1999). Mutations of MIR prevented p150 from binding to HP1α proteins (Murzina et al., 1999). The in vitro and in vivo experiments using GTPase-defective ARL5(Q80L) and GTP-binding-defective ARL5(T35N) mutants of ARL5, as well as ARL5-dMIR, demonstrated GTP-dependent interaction of hARL5 with HP1α that requires its MIR-like motif. All three clones from yeast two-hybrid screening, two full-length and one encoding amino acids 26-191, interacted strongly with ARL5(WT) and ARL5(Q80L), suggesting that the interaction did not involve the N-terminal 1-25 amino acids of HP1α. In the yeast two-hybrid interaction, two regions (residues 1-58 and 158-175) of HP1α were important for ARL5 interaction. Thus, we infer that the interaction of HP1α with ARL5, similar to that of ORC (Pak et al., 1997), involves parts of both the CD and CSD

regions. Unlike other ARLs, ARL4 also contains a MIR-like motif: ^{127}VPVLIV^{132}. However, interaction of hARL4(Q79L) with HP1α is, apparently, much weaker than that of ARL5(Q80L). Three-dimensional structures of mouse HP1 CD and CSD have been published (Ball et al., 1997; Brasher et al., 2000), showing that proteins containing the MIR motif bind to the CSD when HP1 is in a dimeric form (Brasher et al., 2000). Thus, it is conceivable that other domain(s) of ARL5 might also be involved in interacting with HP1 through its CD and CSD regions. ARL5 interacted with all three HP1 paralogues, although the lower binding of HP1β and HP1γ in the two-hybrid assay does not necessarily mean that this also occurs in a mammalian cell context. The HP1 isotype-specificity of ARL5 need to be evaluated further in vivo, during both interphase and mitosis.

The MIR-like motif, ^{128}VPVLVL^{133}, in ARL5, is adjacent to the highly conserved ^{135}NKQD^{138} sequence, which is directly involved in the binding of the guanine nucleotide base. Intriguingly, like ARL5(T35N), ARL5(Q80L/dMIR), in part, is localized to nucleoli, although most of ARL5(Q80L) is found in nucleoplasm, in part associated with HP1α (Fig. 8). It will be important to learn whether deletion of the MIR-like motif from ARL5(Q80L) either directly alters its localization domain(s) or changes the GTP-bound to a GDP-bound conformation, with subsequent localization to nucleoli. Further structural characterization of ARL5 should help to reveal the impact of deletions on the ARL5 folding, perhaps explaining how removal of the MIR-like motif prevents interaction of ARL5 with HP1 and affects subcellular localization of ARL5(Q80L).

Two of the proteins that interacted with ARL5 have been localized to the cytoplasm. One of these is a known GEF (cytohesin-2/ARNO, accession number U70728) for ARF [(Frank et al., 1998; Macia et al., 2001) C.-C. Lee, C.-Y. Lin, J.-C. Kuo and F.-J.S.L., unpublished] and it will be interesting to learn whether it can translocate into nuclei or can activate ARL5. Alternatively, hARL5 may be cytoplasmic, as was hARL5-dC, which had a Golgi-like or punctate distrbution. The small GTPase Ran, which plays a key role in nuclear transport, also functions in mitosis by regulating microtubule nucleation and/or growth (Heald and Weis, 2000). Recently, Ran, in concert with importin α and β, was reported to regulate spindle formation (Gruss et al., 2001; Nachury et al., 2001). The nuclear envelope of higher eukaryotes is a dynamic structure that breaks down during prometaphase, reforming during anaphase and telophase (Gant and Wilson, 1997). During nuclear envelope breakdown, nuclear lamina and pore complexes disassemble, and nuclear membranes vesiculate. During reassembly, nuclear membranes associated with daughter chromosomes fuse to enclose the chromatin. The nucleus then enlarges by importation of proteins through newly assembled pore complexes and fusion of additional vesicles. HP1 could serve as a linker, connecting peripheral heterochromatin to the inner nuclear membrane and mediating nuclear envelope reassembly at the end of mitosis (Ye et al., 1997; Kourmouli et al., 2000). A requirement for a non-ARF GTPase for nuclear fusion and mitotic membrane disassembly was suggested (Gant and Wilson, 1997). We speculate that activated GTP-bound ARL5 may be recruited by HP1 to the heterochromatin regions and have a role in novel nuclear membrane dynamics. It was also suggested that phosphorylation of HP1 proteins can alter their function in the cell cycle and/or development (Zhao and Eissenberg, 1999). Clearly, further studies are necessary to determine the precise role of the interaction between hARL5 and HP1α in embryonic development. To gain new insight into the physiological function of ARL5, we are generating a mouse strain with a targeted deletion of ARL5-coding sequence. We are, of course, interested to determine whether embryonic development is impaired in the ARL5 knock-out mice. These animals should facilitate the identification of additional physiological role(s) of ARL5.

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