A Major Functional Difference between the Mouse and Human ARF Tumor Suppressor Proteins*

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Suppression of tumorigenesis is considerably more stringent in the human than in the much shorter lived mouse species, and the reasons for this difference are poorly understood. We investigated functional differences in the control of the ARF (alternative reading frame) protein that acts upstream of p53 and is encoded along with p16INK4a at a major tumor suppressor locus in both the human and mouse genomes. The mouse and human ARF proteins are substantially divergent at their carboxyl termini. We have shown that the mouse ARF protein (p19ARF) interacts with Pex19p in the cell cytoplasm leading to its nuclear exclusion and repression of its p53 activation function. The human ARF protein (p14ARF) is substantially smaller than its mouse counterpart and is not subject to this functional inactivation by Pex19p. In an identical cellular background, ribozymes directed against Pex19p enhanced p19ARF, but not p14ARF-activated p53 function. This is the first demonstration of a functional difference between the mouse and human ARF proteins. In view of the major role of ARF in tumor suppression, this distinction may contribute to the different levels of tumor proneness of these species.

The INK4a (MTS1, CDKN2) locus on chromosome 9p21 encodes two unrelated tumor suppressor proteins: p16INK4a, an inhibitor of the cyclin D-dependent kinase that acts upstream of the retinoblastoma protein, pRB, and p19ARF, an alternative reading frame protein that acts upstream of p53 (1–8). Each of these proteins has a role in the senescence of primary cells, activates pathways for cell cycle control and tumor suppression (9, 10, 12–15), and is often functionally inactivated in human tumors (8, 16–20). p16INK4a inhibits the activity of cyclin-cyclin D-dependent kinases and thus prevents the phosphorylation and functional inactivation of pRB (21, 22). p19ARF and its human homologue, p14ARF, activate p53 function by restraining the cytoplasmic retention of p19ARF and its downstream p53 activation. This pathway thus provides a regulatory link between pRB and p53 pathways; inactivation of pRb by phosphorylation and release of E2F from pRb-E2F complexes leads to an activation of ARF expression resulting in the cytoplasmic retention of p19ARF and functional dampening of its p53 activation function (36). This effect on p53 is in accord with the proposed involvement of nuclear p19ARF-MDM2 interactions in restraining MDM2-mediated degradation of p53.

Recent studies have shown that ARF function involves complex feedback mechanisms (41, 42). Its expression is regulated by E2F, and thus binding of hypophosphorylated pRB to E2F inhibits ARF expression and its downstream p53 activation. This pathway thus provides a regulatory link between pRB and p53 pathways; inactivation of pRb by phosphorylation and release of E2F from pRb-E2F complexes leads to an activation of ARF expression resulting in the stabilization and functional activation of p53 (5, 43, 44). Activation of the ARF-p53-p21WAF1 pathway can in turn restrict phosphorylation-mediated inhibition of pRB function by inhibiting cyclin-cyclin D-dependent kinase activity.

Functional regulation of ARF is critical for cell cycle control in response to a variety of cellular and environmental signals. The mouse and human ARF proteins share only a limited homology at the cDNA and protein levels (1, 45–47), and the functional relevance of this genetic divergence is unknown. We report here that a result of this difference is that the human ARF protein is not inactivated by Pex19p. This may contribute to the more stringent control of cellular senescence and tumor suppression in human cells.

MATERIALS AND METHODS

Plasmid Construction—Full-length mouse and human Pex19p cDNAs were cloned from mouse and human testis by RT-PCR using mouse sense (5’-gaa ttc atg gcg gct gct gag gaa ggt-3’) and antisense (5’-gtc gac tca cat gat cag aca ttc-3’) and human sense (5’-gaa ttc atg ggc gct gct aga gag ggg-3’) and antisense (5’-gtc gac gca cat gat cag aca ttc-3’) and antisense (5’-gtc gac gca cat gat cag aca ttc-3’). p19ARF-specific primers with EcoRI and SalI sites, respectively. The PCR amplification (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min) product was purified and sequentially ligated to pGEM-T easy (Promega), pOdB8 and pACT2 (yeast two-hybrid vectors (48)), pVP-16 (mammalian two-hybrid vector, CLONTECH), pEGFP-C1 (mammalian

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expression vector for GFP-Pex19p fusion protein, CLONTECH), and pcDNA3/HisMax (mammalian expression vector for HisMax-Pex19p-targeted protein, Invitrogen) vectors. Mouse ARF (p19ARF) and its deletion mutants and human ARF (p14ARF) cDNAs were cloned into pOD8, pACT2, pM (mammalian two-hybrid vector, CLONTECH), and pcDNA3.1 (mammalian expression vector, Invitrogen) vectors by PCR cloning. For expression of hammerhead ribozymes, an expression plasmid (pPUR-KE) containing a chemically synthesized human RNA polymerase III (RNA^{III}) promoter and a puromycin selection marker was used as described (49–51). The integrity of all the plasmids was confirmed by sequencing.

**Yeast Two-hybrid Interactions**—Yeast reporter strain PJ69-2A and Y187 (Trp /Leu /His /Ade ) (48) were transformed with pOD8 plasmid constructs encoding full-length p19ARF and its various deletion mutants or p14ARF. The selected cells were secondarily transformed with the pACT-2-mPex19p or hPex19p constructs. Double transformants that grew on Trp /Leu /His /Ade− selection medium were analyzed for the presence of ARF and Pex19p sequences by PCR and were assayed for β-galactosidase reporter activity. Cell extracts were prepared using standard conditions, and enzyme activity was determined using the GAL-Tropyx kit according to the manufacturer's protocol (Tropix Inc.).

**Cell Culture and Transfections**—Mouse embryonic fibroblasts, NIH 3T3, and monkey kidney cells (COS 7) were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Transfections were performed using LipofectAMINE (Invitrogen). Typically, 1 μg of plasmid DNA was used per well in a 24-well dish, and 3 μg was used per 6-cm dish. For immunostaining, cells were plated on glass coverslips and transfected at 60% confluency. Cells were fixed with methanol:acetone (1:1) at the indicated time intervals following transfections and immunostained as described below. NIH 3T3 cells transfected stably with a construct containing p19ARF cDNA driven by the metallothionein promoter were a kind gift from J. Kato (52). Expression of p19ARF was induced by supplementation of growth medium with 100 μM ZnSO₄. Cells transfected with ribozymes were selected in puromycin-supplemented medium (5 μg/ml for 2 days followed by 0.5 μg/ml for the next 2 days). Expression of ribozymes was detected by RT-PCR, and the effect on Pex19p expression was analyzed by Western blotting.

**Mammalian Two-hybrid Analysis**—COS 7 cells were seeded at 50–60% confluence in 24-well plates and transfected with 1 μg of DNA containing pG5-reporter plasmid, pM/ARF, pVP16/Pex19p, and pM or pVP16 control vectors as indicated in the relevant figure legends. 5 h after transfection, cells were refed with fresh medium and were lysed in universal lysis buffer (Promega) after 48 h. Luciferase activity was measured by using the dual luciferase reporter assay system (Promega). Results presented are the means of at least three transfections.

**In Vivo Coimmunoprecipitation**—Cells transfected with plasmids encoding Myc-targeted ARF proteins and Myc-targeted mevalonate diphosphate decarboxylase (MPD) (an irrelevant control) and GFP-tagged Pex19p or GFP (control). ARF proteins were immunoprecipitated with anti-Myc antibody, and coimmunoprecipitation of Pex19p was detected by Western blotting with anti-GFP antibody. Note that the immunoprecipitation of p14ARF was greater than p19ARF (compare lanes 1 and 2). However, GFP-Pex19p co-precipitated with Myc-ARF (panel b, lane 2) but not with p14ARF (panel b, lane 1) or with MPD (panel b, lane 3). A faint band (close to the size of Pex19p) cross-reacting to GFP antibody was detected in lanes 1–3. GFP by itself showed no coimmunoprecipitation with p19ARF-Myc (panel b, lanes 4–6). Input signals (from 10% of the lysates) for GFP-Pex19p (panel a, lanes 1–3) and GFP (panel a, lanes 4–6) are shown.
mouse ARF protein, p19ARF, by 40 amino acids (43). Notably, a.a. carboxyl-terminal 41 amino acid residues (Fig. 1 data, the Pex19p binding domain of p19ARF was assigned to its mutants that retain the carboxyl-terminal 41 amino acid residues were positive for interactions with Pex19p. Based on these results, the yeast and mammalian two-hybrid assays both suggested that p14ARF does not interact with Pex19p. These findings were confirmed by in vivo co-immunoprecipitation assays (Fig. 2). Whereas immunoprecipitation of p19ARF pulled down Pex19p, an equivalent immunoprecipitation of p14ARF did not (Fig. 2). It was noted that (i) p14ARF runs very close to the dye front on 4–20% SDS-PAGE and (ii) although the amount of immunoprecipitated p14ARF was greater than that of p19ARF from an equal quantity of lysate (Fig. 2, compare lanes 1 and 2), there was no communoprecipitation of Pex19p with p14ARF. This result strongly supported the two-hybrid assays. We therefore concluded that in contrast to p19ARF, p14ARF does not interact with Pex19p.

Because Pex19p was shown to sequester p19ARF in the cytoplasm, we compared the subcellular localization of exogenous Myc-tagged mouse and human ARF proteins in an identical cellular background (HeLa cells). In time course experiments, p19ARF (as detected by staining with anti-Myc antibody) localized first in the cytoplasm (Fig. 3A, a) and subsequently moved to the nucleus and then to the nucleolus (Fig. 3A, b–d). In contrast, p14ARF was visible in the nucleus even at the earliest time point (6 h) (Fig. 3B, b), and as expected, p19ARF, but not p14ARF, colocalized with Pex19p in the cytoplasm (Ref. 36 and data not shown). The cellular background (HeLa cells), the exogenous promoter driving the ARF expression construct, and the level of expression of the two proteins as detected by Western blotting of the transfected cells with anti-Myc antibody (Fig. 3C) were identical in these experiments. Therefore the most likely reason for the different behavior of p14ARF-Pex19p interactions was negative in the yeast two-hybrid system. The mouse and human ARF proteins were also tested for interaction with Pex19p by a mammalian two-hybrid reporter assay (Fig. 1B). The ARF cDNAs were cloned in-frame with the GAL4 DNA binding domain and were expressed in cells along with the DNA activation domain-Pex19p fusion protein. In this assay system, luciferase reporter activity is dependent on the interactions of the DNA binding and activation domains. It detected interaction of Pex19p with p19ARF but not with p14ARF (Fig. 1B). Thus, the yeast and mammalian two-hybrid assays both suggested that p14ARF does not interact with Pex19p. These findings were confirmed by in vivo co-immunoprecipitation assays (Fig. 2). Whereas immunoprecipitation of p19ARF pulled down Pex19p, an equivalent immunoprecipitation of p14ARF did not (Fig. 2). It was noted that (i) p14ARF runs very close to the dye front on 4–20% SDS-PAGE and (ii) although the amount of immunoprecipitated p14ARF was greater than that of p19ARF from an equal quantity of lysate (Fig. 2, compare lanes 1 and 2), there was no communoprecipitation of Pex19p with p14ARF. This result strongly supported the two-hybrid assays. We therefore concluded that in contrast to p19ARF, p14ARF does not interact with Pex19p.

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RESULTS AND DISCUSSION

To elucidate the Pex19p binding domain of p19ARF, β-galactosidase reporter assay (dependent on the interactions of two proteins) was performed on yeast cells transformed with Pex19p and full-length p19ARF and its various deletion mutants. The full p19ARF protein and deletion mutants that retain the carboxy-terminal 41 amino acid residues were positive for interactions with Pex19p. Based on these data, the Pex19p binding domain of p19ARF was assigned to its carboxy-terminal 41 amino acid residues (Fig. 1A, 129–169 a.a.). The human ARF protein p14ARF is shorter than the mouse ARF protein, p19ARF, by 40 amino acids (43). Notably,
FIG. 5. Targeting of Pex19p expression by ribozymes resulted in an activation of p19ARF but not p14ARF function. A, schematic presentation of target sites for Pex19p hammerhead ribozymes. Four target sites flanking the ribozyme cleavage site on Pex19p cDNA sequence and their predicted structures when embedded in tRNA and ribozyme target sequence are shown. B, selection of Pex19p-effective ribozymes. NIH
the mouse and human ARF proteins is their genetic/structural diversity.

It was shown previously that p19ARF-Pex19p interactions result in the dampening of p19ARF function (36). We next compared the biological effects of p19ARF, p14ARF, and p19ARF(d-C41) (a deletion mutant lacking the carboxyl-terminal 41 amino acids) on colony-forming assays. NIH 3T3 cells (which lack endogenous ARF protein (54)) were stably transfected with metal-inducible expression plasmids encoding the above ARF proteins. The selected colonies were analyzed for protein expression by Western blotting, and it was found that there were equal levels of expression of the transfected proteins (similar to the data shown in Fig. 3C). The G418-selected cells were assayed for colony-forming efficiency with or without ARF expression (induced by the addition of 100 μM ZnSO4 into the medium). Expression of p14ARF resulted in 84–87% reduction in the colony-forming efficiency. The expression of p19ARF caused 45–50% reduction (Fig. 4). These results showed that the growth suppressor activity of p14ARF is much stronger than p19ARF. Interestingly, p19ARF(d-C41) had a stronger effect than full-length p19ARF. These results, together with the finding that Pex19p binds to the carboxyl-terminal 40 amino acids of p19ARF and retains it, but not p14ARF, in the cytoplasm (Fig. 3), suggest that p14ARF and p19ARF(d-C41) translocate more rapidly into the nucleus (Fig. 3) resulting in stronger growth suppressor activity.

We next constructed hammerhead ribozymes to target Pex19p expression in NIH 3T3 cells. Target sites flanking the 10 putative ribozyme cleavage sites (GUC, GUA, CUC, and CUA) in the 5' terminus of Pex19p cDNA sequence were selected. Putative structures of each of the target sites along with the ribozyme and the tRNA sequence (155 nucleotides) were predicted using a RNA software (Mulfold2 and LoopViewer) as shown in Fig. 5A. Four target sites (with cleavage sites at nucleotides 40, 108, 122, or 168) that showed at least 60% open structure when embedded in ribozyme and tRNA sequences (Fig. 5A) were selected for construction in the pPUR-KE vector as described (49–51). To select effective ribozymes, NIH 3T3 cells stably expressing His-Max-tagged mouse Pex19p were first made. These were transfected with Pex19p target ribozymes (Fig. 5A). Expression of ribozymes was analyzed by RT-PCR (data not shown), and their effectiveness against Pex19p was analyzed by Western blotting with anti-Xpress RT-PCR (data not shown), and their effectiveness against Pex19p (Fig. 5C). These ribozymes had no effect in the absence of p19ARF. Most notably, these ribozymes did not affect p14ARF-dependent p53 transcriptional activation function (Fig. 5C). Taken together, the results showed that Pex19p interacts with mouse ARF protein and inactivates its function; human ARF by lacking a Pex19p binding region escapes from such inactivation.

ARF is a major tumor suppressor and is involved in cellular senescence (6, 11, 13, 14). It has long been known that there are substantial differences in regulation of cellular senescence in mouse and human cells and that mouse cells become immor-talized much more readily than human cells. The underlying reasons and mechanisms for these differences are not yet understood. We have shown that the ARF protein, a major player in cellular senescence and tumor suppression, is controlled differently in mouse and human cells. Its activity is decreased because of its interactions with Pex19p in the cytoplasm of mouse but not of human cells. This difference may provide a mechanistic explanation of the more stringent imposition of senescence in human cells and their resistance to immortalization.

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