Pex19p Dampens the p19ARF- p53-p21WAF1 Tumor Suppressor Pathway*

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We isolated a 33-kDa protein, Pex19p/HK33/HiPXF, as a p19ARF-binding protein in a yeast two-hybrid screen. We demonstrate here that Pex19p interacts with p19ARF in the cell cytoplasm and excludes p19ARF from the nucleus, leading to a concurrent inactivation of p53 function. Down-regulation of Pex19p by its antisense expression resulted in increased levels of p19ARF, increased p53 function, and a p53/p21WAF1-mediated senescence-like cell cycle arrest. The data demonstrated a novel mechanism of down-regulation of the p19ARF-p53 pathway.

The INK4a (MTS1, CDKN2) locus on chromosome 9p21 is frequently altered in human cancers. It encodes two unrelated tumor suppressor proteins: p16INK4a, an inhibitor of the cyclin-dependent kinases that acts upstream of pRb, and p19ARF, an alternative reading frame protein that acts upstream of p53 (1–3). Both of these proteins have roles in replicative senescence and ras-induced premature senescence of primary cells (4–7). Analysis of p19ARF knock-out mice suggested that this protein functions as a tumour suppressor (3, 8, 9). Recently, it has been shown that p19ARF acts by obstructing degradation and transcriptional silencing of p53 by mdm2 (10, 11). It retains Mdm2 in the nucleolus, preventing its export to the cytoplasm, which is required for mdm2-mediated p53 degradation (12–16). Because p19ARF shares no amino acid homology with known proteins and lacks any decisive functional protein motifs, other cellular factors that might regulate its activity and thereby its execution of growth arrest via the p19ARF-p53 pathway remain poorly defined. Using a yeast interactive screen, we have identified the farnesylated protein Pex19p/HK33/HiPXF (essential for peroxisomal biogenesis) (17–19) as a p19ARF-binding protein. In the present study, we report that the two proteins interact in the cell cytoplasm leading to exclusion of p19ARF from the nucleus and inactivation of p53 function, which constitutes a novel mechanism of down-regulation of the p19ARF-p53 pathway. Neutralization of the Pex19p function by its antisense expression led to an accelerated activation of p19ARF function and p53-p21WAF1-mediated cell cycle arrest that resembled cellular senescence.

MATERIALS AND METHODS

Yeast Two-Hybrid Screen—cDNAs encoding full-length p19ARF (p19ARF-F), amino-terminal 80 amino acids (p19ARF-N) and carboxy-terminal 89 amino acids (p19ARF-C) were cloned into the BamHI, SalI site of the yeast expression vector pODB8 (a kind gift from O. Louvet) (20). For library screening the yeast reporter strain PJ69-2A (Tryp / Leu /His) was sequentially transformed with the plasmid pODB8/ p19ARF and a human testis cDNA library (CLONTECH) according to the manufacturer’s protocol. The cDNA-derived plasmids were recovered from yeast and reintroduced into the yeast reporter strain Y187 to confirm specificity of the interactions. To determine β-galactosidase activity in yeast, five colonies of simultaneously transformed Y187 yeast cells were grown overnight in Leu /Tryp plates. Cell extracts were prepared using standard conditions, and enzyme activity was determined using the GAL-Tropix kit according to the manufacturer’s protocol (Tropix Inc.). The clones were sequenced using an ABI sequencer (PerkinElmer Life Sciences).

Cell Culture and Transfections—Mouse embryonic fibroblasts and monkey kidney cells were cultured in Dulbecco’s modified Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. Transfections were performed using LipofectAMINE™ (Life Technologies, Inc.). Typically, 1 µg of plasmid DNA was used per well of a 24-well dish, and 3µg was used per 6-cm dish.

Plasmid Constructions—Full-length Pex19p was cloned from mouse testis by reverse transcription-polymerase chain reaction using sense (5'-gaa ttc atg gcg gct gct gag gaa ggt-3') and antisense (5'-gtc gac tca cat gat cag aca ctc ttc-3') primers with EcoRI and SalI sites, respectively. Polymerase chain reaction amplification product (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3 min) was purified and sequentially ligated to pGEM-T Easy (Promega), pEGFPC1 (CLONTECH) and pcDNA4/HisMax (Invitrogen) vectors. Mouse p19ARF and its deletion mutants were cloned into the indicated vectors by a similar strategy. The next step was the sequencing of the plasmids confirmed by sequencing.

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/mHK33, VP16/p19ARF, and pM or VP16 control vectors as indicated in the relevant figure legends. After 3 h of transfections, cells were refed with fresh medium and then lysed in universal lysis buffer (Promega) after 48 h. Luciferase activity was measured using the Dual-Luciferase™ reporter assay system (Promega). The results presented are the means of at least three transfections.

In Vivo Immunoprecipitation—Cell lysates (400 µg of protein) in 400 µl of Nonidet P-40 lysis buffer were incubated at 4 °C for 1–2 h with an antibody used for immunoprecipitation, as indicated in figure legends. Immunocomplexes were separated by incubation with protein A/G-Sepharose, Western blotting was performed with the indicated antibodies by standard procedures, and detection was done using ECL chemiluminescence.

Reporter Assays—NIH 3T3, NIH-ARF, NIH-ARFPcDNA4-HisMAX-Pex19p (sense), and NIH-ARFPcDNA4-HisMAX-Pex19p (antisense) derivatives (selected in 1 mg/ml zeocin, Invitrogen) were transfected within the p35-responsive luciferase reporter plasmid, PG-13luc (kindly provided by Dr. Bert Vogelstein). As a control, pRL-TK vector (Promega) was co-transfected in each assay to correct for variations in transfection efficiency. Cells were lysed and measured for luciferase activity as described above.

RESULTS AND DISCUSSION

To isolate p19ARF interacting proteins, a Gal4BD (Gal4 binding domain)-p19ARF fusion protein was used as a bait to screen a library of human cDNAs cloned into a Gal4AD (acti-
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FIG. 1. p19ARF interacts with Pex19p. A, activation of yeast two-hybrid β-galactosidase reporter by Pex19p and either the full-length p19ARF or its carboxy-terminal half. Yeast cells were transformed with plasmids encoding Pex19p and full-length (F), amino-terminal (N), or carboxy-terminal (C) p19ARF, β-galactosidase (β-Gal) activity was measured by liquid assay. B, activation of mammalian two-hybrid luciferase reporter by transfection of cDNAs encoding mouse Pex19p and p19ARF in COS-7 cells. C, in vivo communoprecipitation of Pex19p and p19ARF. Cells were transfected with plasmids encoding a GFP-Pex19p fusion protein or GFP-tesmin (a negative control) and myc epitope-tagged p19ARF (p19ARF-myc) or Mdp-myc (a negative control). Immunoprecipitation was performed with a polyclonal anti-myc antibody, and the myc immunocomplexes were analyzed for the presence of GFP-Pex19p or GFP-tesmin by Western blot analysis with a monoclonal anti-GFP antibody. As a control for the immunoprecipitation, we used C6-36 cells transiently transfected with p19ARF-myc and Mdp-myc. As expected, transfection of these cells with p19ARF resulted in a dose-dependent increase in p53 activity (Fig. 2B, a). Cotransfections of Pex19p with p19ARF blocked p53 activity (Fig. 2B, b). We ruled out the possibility that p53 might be inactivated by direct interaction with Pex19p by performing in vivo communoprecipitation of Pex19p and p53 wherein no Pex19p was seen to precipitate with p53 (data not shown). We next used stably transfected NIH 3T3 cells (NIH-ARF) that express exogenous HA-tagged p19ARF under the control of the heavy metal-inducible metallothionein promoter (22). The addition of 100 μM ZnSO4 to the culture medium resulted in the expression of HA-p19ARF (detectable by Western blotting) and a concurrent increase in p53-dependent luciferase reporter activity (Fig. 2B, c). NIH-ARF cells were stably transfected with expression plasmids encoding sense (S) and antisense (AS) His-tagged Pex19p and were selected in medium supplemented with zeocin (1 mg/ml). These derivatives were analyzed for endogenous p53 activity when cultured in the presence of increasing amounts of ZnSO4. 100 μM induced p19ARF expression detectable by Western blotting. As predicted and consistent with the results of transient transfections (Fig. 2B, b), Pex19p transfectants (NIH-ARF/Pex19p-S) showed down-regulation of p53 function and notably, the antisense derivative (NIH-ARF/Pex19p-AS) showed dramatic up-regulation (Fig. 2B, c). NIH 3T3 cells that lacked p19ARF did not show any effect of transfections of Pex19p and AS constructs on p53-dependent reporter activity (Fig. 2B, d). Taken together these results demonstrated that: (i) an overexpression of Pex19p blocks p19ARF enhancement of p53-mediated transcriptional activation; (ii) this occurs most likely because of nuclear exclusion of p19ARF and abrogation of its interactions with Mdm2, resulting in active degradation of p53; and (iii) such an effect of Pex19p on the transcriptional activity of p53 is mediated by p19ARF.

We next analyzed the effect of Pex19p-p19ARF interactions on the expression of p21WAF1, a gene that is transactivated by p53. Induction of p19ARF led to up-regulation of p21WAF1 expression in NIH-ARF cells as has been described (22). Notably, Western blot analysis of p19ARF and Pex19p with respective tag-specific antibodies revealed that the induction of

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The abbreviations used are: GFP, green fluorescent protein; HA, hemagglutinin; MDP, mevalonate pyrophosphate decarboxylase; ARF, alternative reading frame.
p19ARF led to stabilization of Pex19p (Fig. 3A, a). On the other hand, antisense derivatives of NIH-ARF/Pex19p had more p19ARF than sense derivatives when cultured in the presence of ZnSO4 for an equal time (Fig. 3A, a and b) suggesting that Pex19p may cause destabilization/degradation of p19ARF by a mechanism that remains to be defined. Accordingly, NIH-ARF/Pex19p antisense derivatives showed a high level of p21WAF1 expression, whereas Pex19p sense derivatives showed a lower level as compared with the control NIH-ARF cells (Fig. 3A, a). The induction of p19ARF in NIH-ARF cells by ZnSO4 led to an increase in p53-dependent reporter activity. Expression of Pex19p resulted in a decrease in p53 function, whereas its antisense expression led to a dramatic increase (c). NIH 3T3 cells that lack expression of p19ARF did not show any effect of expression of Pex19p and its antisense construct on p53-dependent reporter activity (d).

As p19ARF and its human homologue p14ARF are important mediators of cellular senescence (5, 6, 9, 23, 24), understanding its precise mechanism of action and how it is controlled is clearly important. p53 has a central role in many aspects of the cell’s response to its environment and control of proliferation (25), in part because of transcriptional control of effectors such as p21WAF1; understanding the factors that regulate its activity is also of critical importance. We have described here a

p19ARF by Pex19p, as demonstrated above. NIH-ARF expressing antisense Pex19p showed severe retardation of growth, exhaustion of their replicative potential, and a senescence-like morphology (Fig. 3, B and C). On the other hand, growth of NIH 3T3 cells that lack p19ARF expression was not affected by transfections of Pex19p-S and -AS, constructs demonstrating that the effect was mediated by p19ARF-Pex19p interactions (Fig. 3C and data not shown). This data implies that p19ARF function is blocked, at least in part, by its interactions with endogenous Pex19p, and abrogation of these interactions by antisense expression of Pex19p led to activation of p19ARF-p53-p21WAF1 pathway and execution of a senescence-like growth arrest.

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novel mechanism of down-regulation of the p19ARF-p53-p21WAF1 pathway.

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FIG. 3. Down-regulation of p19ARF-p53-p21WAF1 pathway by Pex19p. Expression of antisense Pex19p led to activation of the p19ARF-p53-p21WAF1 pathway and induction of a senescence-like phenotype. A, NIH-ARF cells were stably transduced with plasmids encoding sense (Pex19p-S) and antisense (Pex19p-AS) His-tagged Pex19p (His-Pex19p). A high level of expression of Pex19p was seen when cells were induced for p19ARF expression (a). On the contrary, p19ARF was less in NIH-ARF/Pex19p-S cells as compared with either control NIH-ARF or NIH-ARF/Pex19p-AS cells (a and b). Induction of p21WAF1 by p19ARF was less in NIH-ARF/Pex19p-S and more in NIH-ARF/Pex19p-AS as compared with the NIH-ARF (a). Antisense expression of Pex19p neutralized the effects of expressing His-Pex19p in NIH-ARF/Pex19p-S cells (c). B, growth characteristics of NIH-ARF cells stably transfected with Pex19p or its antisense expression construct. Cells maintained in the presence (+) or absence (−) of 100 μM ZnSO4 were fixed, stained with Giemsa and photographed just after the addition of ZnSO4 (Day 0) or after 3 days of culture (Day 3). Note the senescence-like morphology of NIH-ARF/Pex19p-AS cells when cultured in the presence of ZnSO4 C, equal numbers of cells of the types indicated were plated and cultured with (+) or without (−) ZnSO4. Cells were counted in six independent fields after 3 days of culture. Error bars represent standard deviations from six experiments.
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