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PLAGL2 controls the stability of Pirh2, an E3 ubiquitin ligase for p53

Gang Zheng [1], Jinying Ning [2], Yu-Chung Yang *

Department of Pharmacology and Cancer Center, School of Medicine, Case Western Reserve University, 2109 Adelbert Road, W353, Cleveland, OH 44106, USA

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Abstract

PLAGL2 (Pleomorphic Adenoma Gene Like 2) is an oncoprotein involved in various malignancies including lipoblastomas, hepatoblastomas, and acute myeloid leukemia. Although PLAGL2 is known to mainly act as a transcription factor, other functions which may contribute to its oncogenic potential are not clear. Pirh2 (P53 induced RING-H2 protein) is a p53 inducible E3 ligase involved in the ubiquitination of p53, while the mechanisms to regulate its activities are largely unknown. In this study, we show for the first time that Pirh2 forms dimers through its N- and C-terminus in cells and Pirh2 dimers interact with PLAGL2. The interaction between PLAGL2 and Pirh2 dimers prevents proteasomal degradation of Pirh2. This study thus uncovers a novel function of PLAGL2 as an oncoprotein through regulating the stability of Pirh2. Given the importance of Pirh2 in regulating p53 stability, its interaction with PLAGL2 may provide valuable therapeutic targets in treating Pirh2-overexpression malignancies.

Keywords: PLAGL2; Pirh2; Dimer stability

PLAG1 (Pleomorphic salivary Adenoma Gene 1) and PLAGL2 (PLAG1-like 2) are oncogenes involved in various malignancies. Dysregulated PLAG1 expression due to chromosomal translocation is crucial in the formation of pleomorphic adenomas of the salivary glands [1] and other tumors [2–4]. PLAG1 overexpression is also detected in tumors without chromosomal translocation, such as uterine leiomyomas, leiomyosarcomas, and smooth muscle tumors [5]. PLAGL2 is similar to PLAG1 structurally and functionally, and both have been implicated in the pathogenesis of acute myeloid leukemia [6,7]. Similar to PLAG1, PLAGL2 is a transcription factor with a DNA-binding and a transactivation domain [8]. However, besides its role in transcription [9], other functions of PLAGL2 are not well studied.

P53 is important in coordinating cellular responses to stress [10,11]. Given its critical role, it is not surprising that p53 is tightly regulated by post-translational modifications, including ubiquitination [12]. There are several cellular ubiquitination E3 ligases for p53, and their activities are regulated by protein–protein interactions or post-translational modifications. The best studied p53 E3 ligase is Mdm2. If activated in certain malignancies, Mdm2 is able to abolish the tumor suppressor function of p53. There are various regulatory mechanisms to modulate Mdm2 functions. Mdm2 enzymatic activity is inhibited by its association with $p19^{ARF}$ [13], ribosomal protein L11 [14] or TSG101 [15], while enhanced by interacting with MTBP [16] or YY1 [17]. Post-translational modification, such as phosphorylation by Ataxia Telangiectasia-mutated (ATM) in response to DNA damage, also regulates Mdm2–p53 interaction [18]. Other E3 ubiquitination enzymes for p53 are E6-AP [19], COP1 [20], ARF-BP1 [21], and Pirh2 [22]. Among them, Pirh2 is a target gene of p53; its transcript and protein levels increase in response to UV irradiation and cisplatin treatment [22]. A recent study showed that Porcine Circovirus type 2 (PCV2)

* Corresponding author. Fax: +1 216 368 3395.
E-mail address: yxy36@cwru.edu (Y.-C. Yang).
[1] Present address: Department of Pathology, Harvard Medical School, Boston, MA 02115, USA.
[2] Present address: Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA.

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ORF3 protein interacts with Pirh2 and regulates its stability [23]. Despite its importance as a p53 ubiquitination E3 ligase, little is known about how Pirh2 is regulated by cellular factors.

In our study, we found that PLAGL2 interacts with Pirh2 dimers, resulting in its stabilization. This study not only identifies a novel regulatory mechanism for Pirh2, but also provides a mechanistic explanation for PLAGL2's role as an oncoprotein.

Materials and methods

Reagents and antibodies. Anti-Myc (9E10) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-FLAG (M2) was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-HA antibody was from BAbCo (Richmond, CA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C and in 5% CO_2.

Plasmid construction. pcDNA-Pirh2-Myc and other Myc-tagged Pirh2 mutants were constructed by insertion of corresponding cDNA fragments into EcoRI and BamHI sites of pcDNA3.1-Myc-HisB (Invitrogen). GFP-Pirh2 mutants were constructed by insertion of corresponding cDNA fragments into EcoRI and BamHI sites of pEGFP-C1 (Clontech).

Transient transfection, immunoprecipitation, and Western blot analysis. HEK293 cells were transfected by calcium phosphate precipitation method with various plasmid combinations as indicated. Forty-eight hours later, cells were washed with PBS and 1 ml ice-cold lysis buffer (RIPA) (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EGTA, 2 mM Na_3VO_4, 15 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM PMSF) was added. Cells were lysed for 30 min at 4 °C with occasional vortexing. The lysates were collected into 1.5-ml tubes and cleared of nuclei by centrifugation for 10 min at 14,000 rpm. The supernatants (whole cell extracts) were incubated with different antibodies for 16 h at 4 °C and protein A-agarose beads were added for the last hour. The beads were washed five times in TNEN buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 2 mM Na_3VO_4, 1 mM PMSF, and 1 mM NaF). Bound proteins were extracted with SDS–PAGE sample buffer, and analyzed by SDS–PAGE followed by Western blot analysis with the ECL detection system. For two-step immunoprecipitation experiment, whole cell lysates were first incubated with anti-FLAG antibody for 16 h at 4 °C and protein A-agarose beads were added for the last hour. Then the beads were washed four times in 100 mM NaCl buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 0.1% Tween 20). Bound proteins were then eluted four times with 250 μl of 250 μg/ml FLAG peptide (in 100 mM NaCl buffer). In each elution, rock the tubes gently for 15 min and spin 2 min in 2000 rpm. After elution, proceed with anti-HA immunoprecipitation with procedures similar to described above.

Half-life assay. To determine the half-life of Pirh2, HEK293 cells in 6-well plates were transfected with FLAG-Pirh2 expression plasmid, in the presence or absence of HA-PLAGL2. After transfection, cells were treated with 50 μg/ml of cycloheximide for different lengths of time before harvest. Cell lysates were analyzed by immunoblotting with anti-FLAG antibody to detect Pirh2 levels. The data were quantitated with ImageQuant TL v2005.04 software.

Results

Pirh2 forms dimers

Pirh2 was cloned in our laboratory as an interacting protein of Cited2 [CBP/p300 Interacting transactivator with glutamic acid (E) and aspartic acid (D)-rich tail 2] from a yeast two-hybrid screen. Pirh2 has a C2H2-type RING finger domain in the coding region, which is characteristic of E3 ubiquitination ligases. A slow-migrating form of Pirh2 was found when Pirh2-Myc was ectopically expressed in HEK293 cells (Fig. 1A), both in whole cell lysates (lane 1) and in anti-Myc immunoprecipitates (lane 2). When transfected cells were treated with desferrioxamine (DFO), which is an iron chelator, the slow-migrating form of Pirh2 was partially abolished (lanes 3 and 4). These results suggest that the presence of the slow-migrating form of Pirh2 may depend on the availability of iron. Given that the molecular size of Pirh2 is around 30 kDa, and the slow-migrating band is around 60 kDa, we suspected that the SDS-resistant slow-migrating band is the

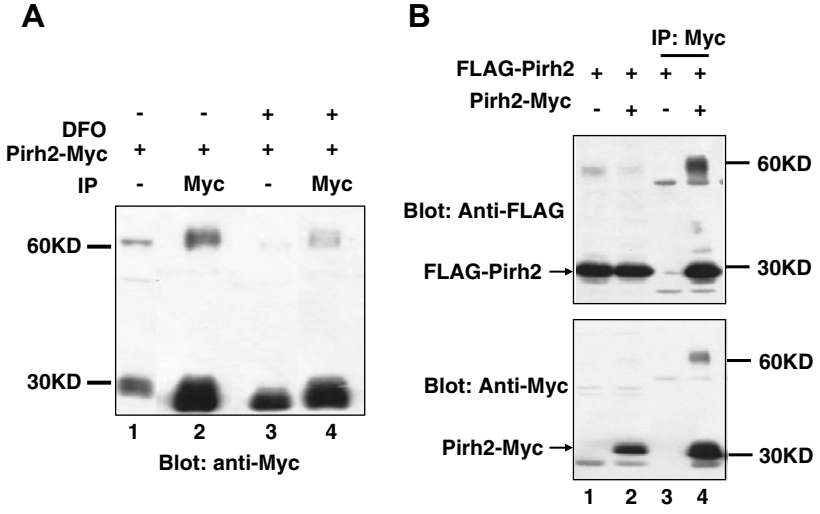

Fig. 1. Pirh2 forms dimers. (A) Two micrograms of Pirh2-Myc was transfected into HEK293 cells using calcium phosphate method. Transfected cells were either left untreated or treated with DFO (20 μM) for 12 h before harvesting. Cell lysates were subjected to immunoprecipitation and Western blot with anti-Myc antibody. All the data in this figure are representatives of at least three independent experiments. (B) Three micrograms of FLAG-Pirh2 was co-transfected with or without 3 μg Pirh2-Myc into HEK293 cells using calcium phosphate method. Cell lysates were subjected to immunoprecipitation with anti-Myc and Western blot with indicated antibodies.