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

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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

1. Introduction

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

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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) 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.

2. Materials and Methods

2.1. 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% CO₂.

2.2. 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).

2.3. 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 Na₃VO₄, 15 µg/ml aprotinin, 10 µg/ml leupeptin, 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 hours 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 Na₃VO₄, 1 mM PMSF, 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 hours at 4°C and protein A-agarose beads were added for the last hour. Then the beads were washed four times in 100mM NaCl buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.1% Tween-20). Bound proteins were then eluted 4 times with 250 µl 250 µg/ml FLAG peptide (in 100mM NaCl buffer). In each elution, rock the tubes gently for 15 mins and spin 2 mins in 2000rpm. After elution, proceed with anti-HA immunoprecipitation with procedures similar to described above.

2.4. 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 μ g/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 was quantitated with ImageQuant TL v2005.04 software.

3. Results

3.1. 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 (lane 3 and lane 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 30KD, and the slow migrating band is around 60KD, we suspected that the SDS-resistant slow migrating band is the dimeric form of Pirh2. To test whether Pirh2 forms dimers, we co-transfected 293 cells with Myc- and FLAG-tagged Pirh2 constructs. As shown in Fig. 1B, immunoprecipitation with anti-Myc antibody pulled down FLAG-Pirh2 only when Pirh2-Myc was expressed (lane 3 and lane 4), suggesting that FLAG-Pirh2 interacts with Pirh2-Myc and Pirh2 forms dimers. Moreover, the slow-migrating form of FLAG-Pirh2 also coprecipitated with Pirh2-Myc (lane 4, upper panel), suggesting that the slow-migrating band on SDS-PAGE is a SDS-resistant dimer of Pirh2. These results show that Pirh2 forms dimers in cells.

3.2. N- and C-terminus of Pirh2 are involved in its dimerization

To map the dimerization region of Pirh2, HEK293 cells were co-transfected with plasmids expressing FLAG-Pirh2 (full-length) and GFP-N-Pirh2 (1–135) or GFP-C-Pirh2 (185–255). As shown in Fig. 2A, both the N- (1–135) and C-terminus (185–255) of Pirh2 were present in the anti-FLAG immunoprecipitates, suggesting that both N- and C-terminus are involved. To further study how Pirh2 forms dimers, we tested whether N-terminus of Pirh2 interacts with N- or C-terminus of Pirh2. HEK293 cells were co-transfected with plasmids expressing Pirh2-Myc (1–135) and GFP-N-Pirh2 (1–135) or GFP-C-Pirh2 (185–255). GFP-N-Pirh2 (1–135) but not GFP-C-Pirh2 (185–255), was present in the anti-Myc immunoprecipitates, suggesting that the N-terminus of Pirh2 interacts with N- but not C-terminus of Pirh2 during dimer formation. These results suggest that both N- and C-terminus are involved and that Pirh2 may form head-to-head dimers in cells.

3.3. PLAGL2 interacts with Pirh2 dimers to stabilize Pirh2

Pirh2 protein sequence was first deposited in protein database as a PLAG1 interacting protein by others (Accession number: AAK96899, unpublished data). We tested whether another member of the PLAG family, PLAGL2, interacts with Pirh2. As shown in Fig. 3A, HA-PLAGL2 expression plasmid was co-transfected with plasmids expressing either full-length Pirh2-Myc or Pirh2 Δ RING-Myc, which lacks the RING finger domain of Pirh2. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA to detect the interaction between PLAGL2 and Pirh2. HA-PLAGL2 coprecipitated with both Pirh2-Myc and Pirh2 Δ RING-Myc, suggesting that the interaction between Pirh2 and PLAGL2 does not

depend on its RING finger domain. Since Pirh2 forms dimers, we next used a two-step immunoprecipitation approach to test whether PLAGL2 interacts with the dimer form of Pirh2. 293 cells were transfected with Pirh2-Myc with or without FLAG-Pirh2 and HA-PLAGL2 and whole cell lysates were first immunoprecipitated with anti-FLAG antibody. After elution with FLAG peptides, second immunoprecipitation was performed with anti-HA antibody. As shown in Fig. 3B, only in the presence of both FLAG-Pirh2 and HA-PLAGL2, Pirh2-Myc could be detected in the final immunoprecipitates, suggesting FLAG-Pirh2, Pirh2-Myc and HA-PLAGL2 are present in the same complex. To further demonstrate that Pirh2 dimerization affects its interaction with PLAGL2, we tested whether FLAG-Pirh2 is able to compete with Myc-tagged Pirh2 for PLAGL2 binding. The rationale behind is that if only Pirh2 monomers interact with PLAGL2, FLAG-Pirh2 will compete with Myc-tagged Pirh2 for PLAGL2 interaction. However, if dimers are involved in the interaction, FLAG-Pirh2 may enhance or inhibit the interaction between Pirh2-Myc and PLAGL2 through the formation of FLAG-Pirh2/Pirh2-Myc dimers, depending on the kinetics and relative levels of each protein. HA-PLAGL2 plasmid was cotransfected with Pirh2-Myc plasmid in the absence or presence of FLAG-Pirh2 plasmid. Cell lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-HA. As shown in Fig. 3C, the interaction between HA-PLAGL2 and Pirh2-Myc was significantly increased when cells co-expressed FLAG-Pirh2, which cannot be explained by the model in which monomeric Pirh2 interacts with PLAGL2. Combined with the two-step immunoprecipitation experiment and the presence of 60KD Pirh2 dimers in cell lysates, these results suggest that Pirh2 dimers are involved in the interaction with PLAGL2.

3.4. PLAGL2 stabilizes Pirh2

To understand the functional significance of Pirh2 interaction with PLAGL2, we first considered whether Pirh2 affects the transactivation activity of PLAGL2, a transcription factor with a transactivation domain at its C-terminus. Transfection studies indicated that Pirh2 does not affect the transactivation function of PLAGL2 (data not shown). Since the Pirh2 level is regulated by ubiquitination [24], and proteins ubiquitinated are expected to exhibit a relatively short half-life due to rapid turnover by the proteasome, we next tested whether PLAGL2 regulates the stability of Pirh2 through their interactions. Cells were transfected with FLAG-Pirh2 plasmid alone or cotransfected with HA-PLAGL2 plasmid, and treated with cycloheximide for different lengths of time before harvest. As shown in Fig. 4A, Pirh2 has a short half-life with its level decreased significantly after 4 hours of cycloheximide treatment. In the presence of PLAGL2, the Pirh2 expression level was significantly increased and the half-life became much longer. As shown in Fig. 4B, quantitation was done with ImageQuant TL v2005.04 software by including actin blot as the loading control. Next we tested whether PLAGL2 stabilizes Pirh2 through inhibition of proteasome-mediated Pirh2 degradation. HEK293 cells were transfected with FLAG-Pirh2 plasmid with or without HA-PLAGL2 plasmid. Cells were either left untreated or treated with MG132 for 6 hours before harvest. As shown in Fig. 4C, MG132 treatment significantly enhanced the Pirh2 level, which was not further increased by PLAGL2 in the untreated cells, suggesting that MG132 abolished the effect of PLAGL2 on Pirh2. These results suggest that PLAGL2 stabilizes Pirh2 through inhibiting proteasome-mediated Pirh2 degradation.

4. Discussion

Pirh2 is one of the E3 ubiquitination enzymes promoting the degradation of p53. In this study, we show that Pirh2 dimerizes and the dimer form of Pirh2 interacts with PLAGL2. Moreover, the interaction with PLAGL2 leads to stabilization of Pirh2, which may contribute to PLAGL2's role as an oncoprotein.

We concluded that Pirh2 forms dimers based on two facts: first, when expressed in cells Pirh2 has both 30KD and 60KD bands; second, Pirh2 with different tags coimmunoprecipitate with each other. However, we could not exclude the possibility that besides dimers, Pirh2 may have other forms of oligomerization. Dimerization is a common mechanism by which the activities of numerous important biological macromolecules are regulated. There are several possibilities for Pirh2 dimer-mediated functions. The first possibility is that the dimer form is important for its interaction with the substrates or interacting partners. The second possibility is that dimerization regulate its E3 ligase activity. Examples of such are CHIP [25] and NOS [26], whose dimerization is required for their enzymatic activities. Another possibility is that dimerization may stabilize Pirh2. A degradation signal may be present in the dimer interface that is sterically blocked in the stable dimeric state. In support of the model, heterodimerization of MAT α 2 and MAT α 1 is known to decrease the ubiquitin-proteasomal degradation of both factors [27], and the stability of NOS is regulated by dimerization [28,29]. This possibility is not mutually exclusive to the others discussed above. That is, the dimerization of Pirh2 may be important for both its stability and enzymatic activity.

Dimerization of enzymes is under tight regulation in cells. A well-studied example is NOS2 (Nitric Oxide Synthase 2). In stimulated macrophages, inactive NOS2 monomers slowly form active homodimers. Many factors are shown to regulate the homodimerization of NOS2. Tetrahydrobiopterin, arginine and heme play critical roles in promoting the homodimerization of NOS2[30,31], while NAPI10 interacts with the NOS2 monomer and inhibits its dimerization and enzymatic activity [32]. We speculate that dimerization of Pirh2 also has complex regulatory mechanisms; in response to different external signals, Pirh2 dimerization and thus its activity may be regulated by different factors. DFO abolished the SDS-resistant dimers of Pirh2 (Fig. 1A) in our study, suggesting that heme may be involved in regulating its dimerization.

Pirh2 has been shown to be overexpressed in lung cancer[33] and act as an oncogene by negatively regulating the p53 level and stability[22]. In our study, PLAGL2 was found to interact with Pirh2 dimers and regulate the stability of Pirh2. Given that Pirh2 negatively regulates p53, PLAGL2 may indirectly regulate p53 through its interaction with Pirh2, and partially contribute to its role as an oncoprotein. Our study also identifies the Pirh2 dimer as a potential target such that chemical compounds or small peptides could be utilized to interfere with Pirh2 dimerization and regulate Pirh2's ability to degrade p53. In cancers with higher expression levels of Pirh2, this strategy is of therapeutic value since it may restore p53 activities.

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Figure 1. Pirh2 forms dimers

A. 2 μ g Pirh2-Myc was transfected into HEK293 cells using calcium phosphate method. Transfected cells were either left untreated or treated with DFO (20 μ M) for 12 hours 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. 3 μ g FLAG-Pirh2 was cotransfected with or without 3 μ g Pirh2-Myc into HEK293 cells using calcium phosphate method. Cell lysates were subjected to immunoprecipitation with anti-Myc and Western blot with indicated antibodies.

A **B**

Figure 2. N- and C- terminus of Pirh2 are involved in dimerization

A. 3 μ g FLAG-Pirh2 plasmid was cotransfected with 3 μ g GFP-N-Pirh2 (1–135) or GFP-C-Pirh2 (185–255) plasmid. Cell lysates were subjected to immunoprecipitation with anti-FLAG and Western blot with anti-GFP and anti-FLAG. All the data in this figure are representatives of at least three independent experiments.

B. 3 μ g N-Pirh2 (1–135)-Myc plasmid was cotransfected with 3 μ g GFP-N-Pirh2 (1–135) or GFP-C-Pirh2 (185–255) plasmid. Cell lysates were subjected to immunoprecipitation with anti-Myc and Western blot with anti-GFP and anti-Myc.

A

B

Pirh2-Myc	+	+	+
FLAG-Pirh2	-	+	+
HA-PLAGL2	-	-	+

Blot: Anti-Myc **Blot: Anti-FLAG** **Blot: Anti-HA** **IP: FLAG
Blot: Anti-Myc** **IP: FLAG
Blot: Anti-HA** **1st IP: FLAG 2nd IP: HA
Blot: Anti-HA** **1st IP: FLAG 2nd IP: HA
Blot: Anti-Myc**

C

Figure 3. PLAGL2 interacts with Pirh2 dimers

A. 3 μ g HA-PLAGL2 plasmid was cotransfected with 2 μ g Pirh2-Myc or Pirh2 Δ RING-Myc plasmid. 48 hours after transfection, cells were harvested. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and Western blot with antibodies indicated. All the data in this figure are representatives of at least three independent experiments.

B. 3 μ g Pirh2-Myc plasmid was cotransfected with 3 μ g FLAG-Pirh2 plasmid in the presence or absence of 3 μ g of HA-PLAGL2 plasmid. 48 hours after transfection, cells were harvested. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody first, then eluted with FLAG-peptides. Second immunoprecipitation was performed with anti-HA antibody and analyzed by Western blot with antibodies indicated.

C. 3 μ g HA-PLAGL2 plasmid was cotransfected with 2 μ g Pirh2-Myc plasmid in the presence or absence of various amounts (0, 2 and 6 μ g) of FLAG-Pirh2 plasmid. 48 hours after transfection, cells were harvested. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody, and Western blot with antibodies indicated.

Figure 4. PLAGL2 stabilizes Pirh2

A. 0.5 μ g FLAG-Pirh2 plasmid was transfected into HEK-293 cells (6-well plates) in the absence or presence of 1 μ g HA-PLAGL2 plasmid. Cells were treated with 50 μ g/ml cycloheximide for different lengths of time before harvest. Cell lysates were analyzed by Western blot with indicated antibodies. All the data in this figure are representatives of at least three independent experiments.

B. Quantitation of the data from Fig. 4A. Using ImageQuant TL v2005.04 software, quantitation was done by comparing signal ratio of Pirh2 and actin at each time point.

C. 0.5 μ g FLAG-Pirh2 plasmid was transfected into HEK-293 cells (6-well plates) in the absence or presence of 1 μ g HA-PLAGL2 plasmid. 0.2 μ g GFP-expressing plasmid was used as an internal control. Cells were left untreated or treated with 20 μ g/ml MG132 for 6 hours before harvest. Cell lysates were analyzed by Western blot with indicated antibodies.