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The pathway for IRP2 degradation involving 2-oxoglutarate-dependent oxygenase(s) does not require the E3 ubiquitin ligase activity of pVHL ☆

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Abstract

Iron regulatory protein 2 (IRP2), a posttranscriptional regulator of iron metabolism, is subjected to iron-dependent degradation by the proteasome. Recent experiments proposed a mechanism involving 2-oxoglutarate-dependent oxygenases. Enzymes of this class, such as prolyl-4-hydroxylases, mediate the oxygen and iron-dependent degradation of the hypoxia inducible factor HIF-1α, which requires the E3 ubiquitin ligase activity of pVHL. Considering that the pathways for IRP2 and HIF-1α degradation share remarkable similarities, we investigated whether pVHL may also be involved in the degradation of IRP2. We show here that IRP2 can interact with pVHL in co-transfection/co-immunoprecipitation assays. Furthermore, pVHL is able to promote the ubiquitination and the decay of transfected IRP2. However, the iron-dependent degradation of endogenous IRP2 is not impaired in VHL-deficient cell lines, suggesting that pVHL is not a necessary component of this pathway.

Keywords: Iron regulatory protein; Iron-responsive element; Transferrin receptor; Ferritin

1. Introduction

Iron regulatory protein 2 (IRP2) and its close homologue IRP1 are important regulators of cellular iron homeostasis. They bind to "iron responsive elements" (IREs), structural motifs in the untranslated regions of several mRNAs encoding proteins of iron metabolism, and thereby control their translation or stability [1,2]. Among others, the IRE/IRP system regulates the expression of transferrin receptor 1 and ferritin, which mediate cellular iron uptake and storage, respectively. The targeted disruption of mouse IRP2 has been associated with aberrant iron homeostasis in the intestinal mucosa and the CNS, and with the development of a neurodegenerative disorder [3], suggesting that IRP2 has a critical function in the context of systemic iron metabolism.

IRP2 is regulated in response to iron and oxygen supply at the level of protein stability. It remains stable in iron-starved or hypoxic cells; however, it undergoes degradation by the proteasome following iron or oxygen administration [4,5]. A mechanistic model postulated that the signal for IRP2 recognition by the proteasome is the site-specific oxidation of three cysteine residues within a 73-amino-acid "degradation domain" [6,7]. We [8] and others [9] recently demonstrated that this domain is dispensable for IRP2 regulation. We also showed that the pathway for IRP2 degradation is saturable [8], an observation that may help to reconcile previous data. Moreover, antioxidants, such as ascorbate, α-tocopherol and *N*-acetyl-cysteine, promote the degradation of IRP2 [8].

Abbreviations: IRP2, iron regulatory protein 2; IRE, iron-responsive element; HIF, hypoxia inducible factor; pVHL, von Hippel–Lindau protein; GFP, green fluorescent protein; FAC, ferric ammonium citrate; DFO, desferrioxamine; DMOG, dimethyl-oxalyl-glycine; IP, immunoprecipitation

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The saturation of the IRP2 degradation machinery and the effects of iron, oxygen and ascorbate are strongly reminiscent of the mechanism for degradation of the hypoxia inducible factor 1α (HIF-1α) [8,10], which mediates the hypoxic response [11,12]. The first step in the pathway for HIF-1α degradation is the hydroxylation of P402 and P564 [13]. This crucial posttranslational modification provides a recognition site for the von Hippel–Lindau tumor suppressor protein (pVHL), a component of ubiquitin ligase complex [14–16]. The reaction is catalyzed by the prolyl-4-hydroxylases PHD1, PHD2 and PHD3 [17], enzymes belonging to the family of 2-oxoglutarate-dependent oxygenases that utilize oxygen, iron and ascorbate as cofactors [18].

Recent data [8,9] showed that the iron-dependent degradation of IRP2 in previously iron-depleted cells can be efficiently antagonized by dimethyl-oxalyl-glycine (DMOG), an inhibitor of 2-oxoglutarate-dependent oxygenases. These results define a novel pathway for IRP2 degradation, involving the activity of 2-oxoglutarate-dependent oxygenases, and raise the question whether IRP2 and HIF-1α utilize pVHL as a common E3 ubiquitin ligase. Here we examine a possible role of pVHL on IRP2 degradation.

2. Materials and methods

2.1. Materials and plasmids

Ferric ammonium citrate (FAC) and MG132 were purchased from Sigma (St. Louis, MI). Desferrioxamine (DFO) was from Novartis (Dorval, Canada). The pcDNA3-VHL-FLAG and pcDNA3-His-Ub plasmids, encoding FLAG-tagged pVHL and ubiquitin, respectively, were kindly provided by Dr. Antonis Koromilas (Montreal, PQ). The pcDNA3-IRP2-HA plasmid, encoding HA-tagged IRP2, was described in Ref. [8]. The pEGFP-N3 plasmid encoding the enhanced green fluorescent protein (GFP) was from Clontech.

2.2. Cell culture and transfections

VHL-deficient 786-O and RCC4 (human renal carcinoma) cells, VHL stable transfectants 786-O/VHL [19] and RCC4/VHL [20], and H1299 (human lung cancer) cells were maintained in supplemented DMEM. Transient transfections in 786-O cells were performed with the vaccinia virus/T7 RNA polymerase hybrid system [21], and in H1299 cells with the Lipofectamine Plus™ reagent (Gibco BRL).

2.3. Co-immunoprecipitation (IP) assays and Western blotting

The cells were lysed in a buffer containing 1% Triton X-100, 50 mM Tris–Cl pH 7.4, 150 mM NaCl and a cocktail of protease inhibitors (1 μg/ml of aprotinin, leupeptin and pepstatinA, and 50 μg/ml phenylmethanesulfonyl fluoride). Cell debris was cleared by centrifugation and cell lysates (250 μg) were subjected to quantitative IP in a total volume of 800-μl lysis buffer with 1-μg HA (Santa Cruz) or M2-FLAG (Sigma) antibodies. The immunoprecipitated proteins or total cell lysates (30 μg) were analyzed by SDS-PAGE on 10% gels, followed by Western blotting as described in Ref. [8]. Dilutions were 1:1000 for HA, FLAG, ubiquitin (Santa Cruz), β-actin (Sigma) and IRP2 [22] antibodies, and 1:500 for pVHL (Pharmingen) and GFP (Invitrogen) antibodies, respectively.

2.4. Pulse chase

The cells were metabolically labeled for 2 h with (50 μCi/ml) Trans-[^{35}S]-label, a mixture of 70:30 ^{35}S-methionine/cysteine (ICN), and chased with cold media for different time intervals. Cell lysates were analyzed by IP with the HA antibody as in Ref. [8].

3. Results and discussion

3.1. pVHL interacts with co-transfected IRP2

Previous experiments showed that pVHL directly interacts with HIF-1α [23]. To examine whether pVHL may also possess the potential to interact with IRP2, plasmids encoding FLAG-tagged pVHL and HA-tagged IRP2 were co-transfected into VHL-deficient 786-O cells. The expression of chimeric proteins was driven by the vaccinia virus/T7 RNA polymerase hybrid system. A possible interaction between pVHL and IRP2 was assessed by a co-IP assay. The experiment depicted in Fig. 1A shows that HA-tagged IRP2 can be readily detected by an immunoblot with HA antibodies in pVHL-FLAG immunoprecipitates (lanes 4–6), and vice versa (lanes 7–9). The input is indicated by analysis of the total cell extracts by Western blotting with HA or pVHL antibodies (lanes 1–3).

It should be noted that chimeric proteins driven by the vaccinia virus/T7 RNA polymerase hybrid system are expressed at very high levels. Based on normalization of transfected IRP2 with endogenous β-actin [8], we estimated that this system yields ~60–80-fold higher levels of protein expression compared to lipofectamin transfection. We therefore addressed whether the pVHL/IRP2 interaction persists under conditions where these proteins are only modestly overexpressed. To this end, plasmids encoding pVHL-FLAG and IRP2-HA or control GFP were transiently co-transfected in H1299 cells, and protein–protein interactions were analyzed in cell extracts by the co-IP assay as described above. Under these conditions, pVHL-FLAG does not form a complex with control GFP (Fig. 1B), but promptly interacts with IRP2-HA (Fig. 1C). We conclude that chimeric pVHL and IRP2