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The central domain of yeast transcription factor Rpn4 facilitates degradation of reporter protein in human cells

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ABSTRACT

Despite high interest in the cellular degradation machinery and protein degradation signals (degrons), few degrons with universal activity along species have been identified. It has been shown that fusion of a target protein with a degradation signal from mammalian ornithine decarboxylase (ODC) induces fast proteasomal degradation of the chimera in both mammalian and yeast cells. However, no degrons from yeast-encoded proteins capable to function in mammalian cells were identified so far. Here, we demonstrate that the yeast transcription factor Rpn4 undergoes fast proteasomal degradation and its central domain can destabilize green fluorescent protein and Alpha-fetoprotein in human HEK 293T cells. Furthermore, we confirm the activity of this degron in yeast. Thus, the Rpn4 central domain is an effective interspecies degradation signal.

1. Introduction

The turnover of intracellular proteins is a tightly regulated and constantly adjusted process, with the 26S proteasome complex as one of the key players. In most cases, protein substrates are tagged with ubiquitin (Ub) chains that are recognized by the 19S regulatory particle of the 26S proteasome. On the other hand, increasing numbers of proteins are shown to be degraded by the proteasome without prior ubiquitination [4]. Among these: ornithine decarboxylase (ODC), thymidylate synthase, yeast Rpn4, c-jun, p-21 and others [15,10,8,18]. Furthermore, it is estimated that up to 22% of all cellular proteins undergo proteasome-dependent proteolysis when omitting ubiquitination [3]. Special motifs denoted as degradation signals (degrons) are responsible for ubiquitin-dependent and ubiquitin-independent degradation [28]. Ubiquitin-dependent degrons consist of a preferential ubiquitination site (mostly lysine) and a distinct sequence. Conversely, ubiquitin-independent degradation signals frequently share structural, rather than sequence, similarities. They contain an intrinsically disordered region and an alpha helix, which serve two major functions: the initiation of degradation and tethering to the proteasome, respectively [17,21]. Little is known about interspecies degrons and the evolution of the degradation machinery from lower to higher eukaryotes. The rare example, except classical N-degrons [25], is the mammalian ODC degron, which promotes the degradation of chimeric proteins in mammalian and yeast cells [5,23,11,19]. However, no yeast-derived, degradation signals, capable to work in mammalian cells have been discovered. Yeast protein Rpn4 (yRpn4) is a transcription factor with extremely short half-life (1–2 min). It controls proteasome abundance through a negative feedback mechanism [16,27]. Turnover of yRpn4 plays an important role in the resistance to various stresses [26,12] and is tightly regulated. It was shown that degradation of yRpn4 can be both Ub-dependent and -independent [10]. The N-terminal portion of yRpn4 (a.a. 1–80) contains a portable Ub-independent degron with an intrinsically disordered domain and a folded segment [7] (Fig. 1A and Suppl. Fig. 1A). This degron mediates the cotranslational degradation of yRpn4 [6]. Portable ubiquitin-dependent degron of yRpn4 was mapped to 172–229 a.a. [9]. It contains the preferable polyubiquitination site K187 and proximal acidic domain that mediates the interaction with the cognate ubiquitin ligase Ubr2 [9] (Fig. 1A).

Given that yRpn4 degradation is mediated by the evolutionarily conservative components of the ubiquitin–proteasome system, we suggested that yRpn4 could be highly unstable in higher eukaryotic cells and contain degrons capable of promoting chimeric protein degradation in mammalian cells. In this study, we demonstrated that human proteasomes effectively degrade yRpn4 as well as two different stable proteins fused to the central domain of yRpn4.

Abbreviations: ODC, ornithine decarboxylase; GFP, green fluorescent protein; AFP, Alpha-fetoprotein; Ub, ubiquitin; CMV, cytomegalovirus

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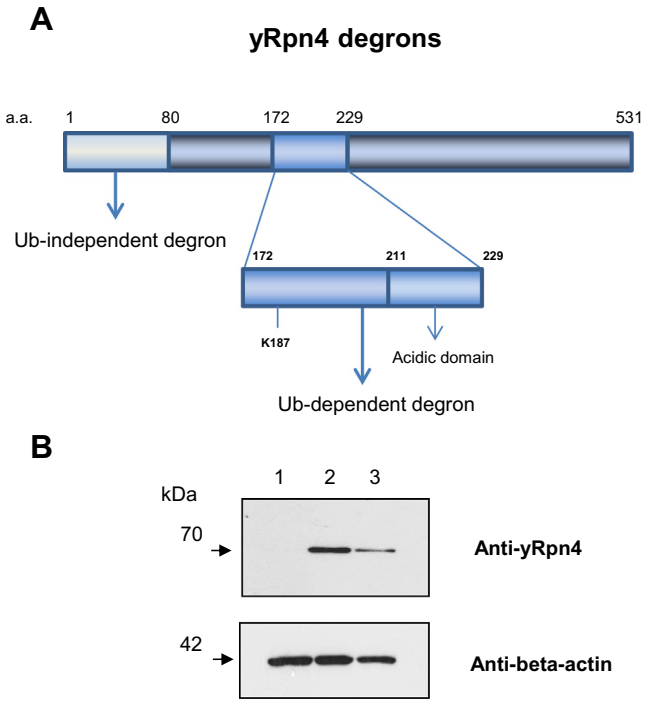

Fig. 1. Recombinant yRpn4 accumulates in transfected HEK 293T cells after treatment with proteasome inhibitor. Schematic representation of yRpn4 degrons (A). Lysine 187 is marked. (B) Proteasome inhibition assay. Western blot analysis of lysates of transfected 293T cells. Cells were maintained in growth media containing no proteasome inhibitor (track 1), 10 μM MG132 or 10 μM lactacystin (tracks 2 and 3 respectively). yRpn4 was detected by Western blotting with anti-yRpn4 antibodies. Equal protein loading was confirmed by staining the membrane with anti-β-actin antibodies. yRpn4 and β-actin are marked by arrow heads.

2. Materials and methods

2.1. Cell lines

The human embryonic kidney cell line HEK 293T (ATCC CRL-3216) was used for transfection. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, L-glutamine and antibiotics (all from PanEco, Russia) and kept at 37 °C, 5% CO_2 and 95% humidity.

2.2. Vectors

Using the pEGFP-N1 (Clontech, USA) backbone, two eukaryotic expression vectors encoding the yRpn4 and GFP-ODC degradation signal chimera were designed. The *RPN4* gene and mouse ODC degradation signal were cloned from previously obtained plasmids [13,19], and the constructs were denoted pyRpn4 and pGFP-ODC-signal. The design of additional control vectors pΔAFP (encoding murine Alpha-fetoprotein (AFP) without leader sequence) and pΔAFPODCsignal (encoding murine Alpha-fetoprotein without leader sequence and carrying ODC degron) is described elsewhere [20,19]. The leader sequence was removed in order to ensure cytoplasmic localization of the recombinant proteins.

Plasmids pyRpn4(1–82)-N-GFP, pyRpn4(1–110)-N-GFP, pyRpn4(1–176)-N-GFP, pyRpn4(172–229)-N-GFP, pyRpn4(177–327)-N-GFP and pyRpn4(230–327)-N-GFP were obtained by cloning in-frame *RPN4* gene fragments encoding yRpn4 portions (the corresponding amino acids are indicated in brackets) upstream of the *GFP* gene in pEGFP-N1. Plasmids pGFP-C-yRpn4(1–82), pGFP-C-yRpn4(1–110), and pGFP-C-yRpn4(172–229) were obtained by cloning the *RPN4* fragments downstream of the *GFP* gene in pEGFP-N1. Plasmid pyRpn4(177–327)-N-ΔAFP was derived from pΔAFP by cloning *RPN4* gene fragment upstream of the *ΔAFP* gene. The constructs were verified by restriction analysis and sequencing. Plasmids were amplified in DH5α cells and isolated using the Qiagen Plasmid Mini kit (Qiagen, Germany). All proteins encoded by these vectors bore a stabilizing amino acid at their N-termini next to methionine to avoid degradation by the N-terminal rule.

2.3. Transfection and preparation of cellular lysates

HEK 293T cells (3×10^5) were transfected with 1 μg of plasmids using Mirus TransIT-293 reagent (Mirus Bio, USA) according to the manufacturer's instructions. 48 h post transfection cells were first examined under a DMI 4000B fluorescent microscope (Leica, Germany) and then washed, scraped and analyzed by flow cytometry or lysed in NP-40 buffer (Tris–HCl, pH 8.0 50 mM, NaCl 150 mM, NP-40 1%, EDTA 5 mM, containing protease inhibitor cocktail (Roche, Germany)). Lysates were collected and stored at −80 °C before use.

2.4. SDS PAGE and Western blotting

Proteins were separated in 10% or 13% Tris–Glycine polyacrylamide gels followed by transfer onto nitrocellulose membranes (BioRad, USA). The GFP and GFP-yRpn4 chimeras were detected using polyclonal rabbit anti-GFP antibodies (Abcam, USA) taken 1:1000 or mouse monoclonal anti-GFP antibodies taken 1:1000 (ProteinSynthesis, Russia) and goat anti-mouse, or anti-rabbit HRP conjugated antibodies, both were used 1:20,000 (Enzo, USA and Abcam, USA respectively). AFP was detected using polyclonal rabbit anti-AFP antibodies diluted 1:1000 and goat anti-rabbit HRP conjugates (both from Abcam, USA). To stain yRpn4, we used rabbit anti-yRpn4 antibodies [14]. Ubiquitin was revealed using rabbit anti-Ub antibodies taken 1:1000 (Abcam, USA). The blots were detected using ECL kit (GE Healthcare, UK). To ensure equal loading of protein in each lane, the membranes were stripped and stained with anti-β-actin antibodies diluted from 1:1000 to 1:2000 (Sigma–Aldrich, Germany).

2.5. Proteasome inhibition assay with MG132/lactacystin and cycloheximide/puromycin chase

Thirty hours post transfection, HEK 293T cells were treated with 10 μM MG132/lactacystin (both from Sigma–Aldrich, Germany) and incubated for 18 h at 37 °C. Treated cells were then examined using a DMI 4000B fluorescent microscope (Leica, Germany), after that, samples were investigated by flow cytometry or lysed and analyzed by Western blotting.

The translation inhibitors cycloheximide (CHX, Sigma–Aldrich, Germany) and puromycin (Sigma–Aldrich, Germany) were used to evaluate the kinetics of recombinant protein degradation; 48 h post transfection, HEK 293T cells were treated with 100 μg/ml (final concentration) cycloheximide or 10 μg/ml puromycin. Immediately after treatment and after 2, 4 and 6 h of incubation with the translation inhibitors, the cells were scraped and lysed, and the cellular lysates were analyzed by Western blotting. Experiments were performed twice.

2.6. Accumulation chase

Forty-eight hours post transfection, the cell culture media was supplemented with MG132 (10 μM); and after incubation for 2, 4 and 6 h, the cells were lysed and analyzed for recombinant protein accumulation by Western blotting. The signals were normalized by staining the membrane with anti-β-actin antibodies (Sigma–Aldrich, Germany). X-ray films with the detected bands

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