FEBS Letters 584 (2010) 67–73

FEBS Letters

journal homepage: www.FEBSLetters.org

Direct proteasome binding and subsequent degradation of unspliced XBP-1 prevent its intracellular aggregation

Ami Navon [a], Ariel Gatushkin [b], Lior Zelcbuch [a], Shimon Shteingart [b], Marganit Farago [c], Rivka Hadar [b], Boaz Tirosh [b,*]

[a] Department of Biological Regulation, The Weizmann Institute of Science, Rehovot, Israel
[b] The Institute of Drug Research, The School of Pharmacy, The Hebrew University, Jerusalem, Israel
[c] Lautenberg Center for General and Tumor Immunology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel

ARTICLE INFO

Article history:
Received 9 September 2009
Revised 18 November 2009
Accepted 19 November 2009
Available online 24 November 2009

Edited by Noboru Mizushima

Keywords:
Unfolded protein response
ER stress
XBP-1
Proteasome
Ubiquitin
In vitro degradation

ABSTRACT

The non-canonical splicing of XBP-1 mRNA is a hallmark of the mammalian unfolded protein response (UPR). The proteasomal degradation of unspliced XBP-1 (XBP-1u) facilitates the termination of the UPR. Thus, understanding the mechanism of XBP-1u degradation may allow control over UPR duration and intensity.

We show that XBP-1u interacts with purified 20S proteasomes through its unstructured C-terminus, which leads to its degradation in a manner that autonomously opens the proteasome gate. In living cells, the C-terminus of XBP-1u accumulates in aggresome structures in the presence of proteasome inhibitors. We propose that direct proteasomal degradation of XBP-1u prevents its intracellular aggregation.

Structured summary:
MINT-7302217: *XBP1-u* (uniprotkb:P17861-1) *binds* (MI:0407) to *Proteasome subunit alpha 7.2* (uniprotkb:O14818) by *pull down* (MI:0096)
MINT-7302148: *Vimentin* (uniprotkb:P08670) and *XBP1-u* (uniprotkb:P17861-1) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-7302163: *XBP1-u* (uniprotkb:P17861-1) *binds* (MI:0407) to *Proteasome subunit alpha 5* (uniprotkb:P28066) by *pull down* (MI:0096)
MINT-7302186: *XBP1-u* (uniprotkb:P17861-1) *binds* (MI:0407) to *Proteasome subunit alpha 6* (uniprotkb:P60900) by *pull down* (MI:0096)

1. Introduction

The endoplasmic reticulum (ER) is the port of entry of proteins into the secretory pathway. Eukaryotic cells developed mechanisms to adjust the amount of secretory pathway components to meet the cellular demand. This is driven by conditions of ER stress, which ensue when the amount of client proteins that emerge into the ER exceeds its overall folding capacity. ER stress in eukaryotic cells activates an intricate ER-to-nucleus signaling cascade that is collectively termed the unfolded protein response (UPR) [1], which is required for normal embryonic development [2–4], and its dysregulation in adulthood may cause a wide range of disorders [5–9].

The mammalian UPR is composed of three transducers: PKR-like ER eIF2α kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1). While the PERK and ATF6 pathways are unique to mammalian cells, the IRE1 pathway is conserved among all metazoans. Upon ER stress, IRE1 cleaves the mRNA of X-box-binding protein 1 (XBP-1). This non-canonical splicing converts the unspliced XBP-1 (XBP-1u) into the spliced form (XBP-1s) [10–12]. XBP-1s is a potent transcription factor that promotes the transcription of a large number of target genes [13–15].

In yeast, the orthologue of XBP-1, Hac1, is not translated in its unspliced form [16]. XBP-1u, however, is constantly generated, but undergoes rapid proteasomal degradation [10,11], which limit its biological significance. Nonetheless, several roles for XBP-1u have been proposed. In *Caenorhabditis elegans* XBP-1u has a direct role in transcription [15]. In mammalian cells, XBP-1u serves as a positive and negative modulator of the UPR. On the one hand it promotes the targeting of its own mRNA to the ER membrane, which facilitates the encounter with IRE1 and accelerates the

Abbreviations: AMC, 7-amido-4-methylcoumarin; ATF6, activating transcription factor 6; ER, endoplasmic reticulum

* Corresponding author. Address: The School of Pharmacy, The Hebrew University, P.O. Box 12065, Jerusalem 91120, Israel. Fax: +972 2 6758741.
E-mail address: boazt@ekmd.huji.ac.il (B. Tirosh).

0014-5793/$36.00 © 2009 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2009.11.069

splicing [17]. On the other hand, XBP-1u facilitates the proteasomal degradation of ATF6 and XBP-1s. XBP-1u shuttles between the nucleus and cytoplasm [18]. It was demonstrated that XBP-1u heterodimerizes with XBP-1s or ATF6 in the nucleus, which allows the subsequent trafficking of the dimer to the cytoplasm where it undergoes degradation as a complex [18,19].

We previously showed that the C-terminus of XBP-1u (XBP-1^{CTu}) acts as a potent degron when appended onto otherwise stable proteins, such as GFP or a HA-tag concatamer [20]. Our data inferred that this proteasomal degradation may not require its prior ubiquitination, however no direct evidence was provided.

Here, we directly addressed the requirement of ubiquitination for XBP-1u degradation in vitro. Our data show that XBP-1^{CTu}, but not its N-terminus (the domain shared by XBP-1u and XBP-1s, referred to as XBP-1^{NT}), interacts with, the 20S proteasomes and undergoes degradation. Interestingly, in living cells, upon proteasome inhibition, XBP-1u has a strong propensity to aggregate and accumulates in the aggresome. We propose that direct proteasomal degradation may be a preemptive measure against XBP-1u aggregation.

2. Materials and methods

2.1. Cells and reagents

293T cells were maintained in DMEM (10% FCS, 2 mM glutamine, 50 U/mL penicillin and 50 μg/mL of streptomycin). Cells were transfected using standard calcium phosphate precipitation protocol. ZL_3VS was kindly provided by Dr. Hidde Ploegh (WIBR, MA). Anti-His tag antibodies were purchased from Santa Cruz biotechnology. 20S and 26S proteasomes were purified from rabbit muscle.

2.2. Degradation of XBP-1 by the proteasome: preparation of substrates

XBP-1u and XBP-1^{NT} fused to a C-terminal 6XHis tag were cloned into pIVEX2.3d. BL21 bacteria were transformed with the vectors and protein expression was induced by IPTG (0.5 mM) for 16 h at 16 °C. After resuspension in HEPES 20 mM (pH 7.5) and sonication, lysates were cleared by centrifugation (10 000×*g*, 10 min, 4 °C). Pellets were dissolved in 6 M guanidinium chloride. Proteins were purified over Ni^+ columns followed by extensive washing with 1 M NaCl, 20 mM HEPES (pH 7.5), 1% Tween20. Proteins were eluted by 2 M imidazole and dialyzed against 50 mM HEPES, 50% glycerol. The final concentration of XBP-1u was approximately 100 μg/mL, while for XBP1-DN it was 50 μg/mL. Purity was 90% (determined by SDS–PAGE).

2.3. Proteasomal degradation reaction conditions and analysis

Proteasomes were purified as previously described [21]. XBP-1u and XBP-1^{NT} were incubated at 37 °C in HEPES buffer (50 mM HEPES pH 7.5, 1 mM DTT with or without 0.018% SDS) in the presence of 20S proteasomes. For degradation by 26S proteasomes, the proteins were incubated without SDS. For all degradation reactions the molar ratio of proteasomes to substrates was 1:1000 (1 nM 20S and 26S proteasomes, 1 μM substrate). Samples from reaction mixtures were withdrawn periodically and subjected to immunoblot analysis with an anti-His tag. The immunoblots were quantified using Quantity One program (Bio-Rad).

2.4. LLVY-AMC proteasomal hydrolysis

Purified 20S proteasomes were incubated at a final concentration of 16 nM with 100 μM fluorogenic 7-amido-4-methylcoumarin (AMC) tetrapeptide substrates Suc-LLVY-AMC (Bachem) in the presence and absence of XBP-1^{NT} or XBP-1u (50 nM). AMC fluorescence was measured in 96-well plates equilibrated to 37 °C. Reaction was terminated after 20 min. Excitation wavelength was 370 nm; emission was recorded at 465 nm.

2.5. On-beads interactions with XBP-1u

XBP-1u and XBP-1^{NT} were cloned into pGEX6p-1 vectors in frame to GST. Competent BL21 bacteria were transformed with the vectors and protein expression was induced by IPTG for 4 h at 37 °C. Bacteria were lyzed in STE lysis buffer (NaCl 150 mM, Tris (pH 8) 10 mM, EDTA 1 mM), which contained 1.5% of sodium lauryl sarcozine. Following sonication and clearing by centrifugation (10 000×*g*, 30 min, 4 °C) lysates were loaded onto glutathione beads. Beads were incubated with lysates (90 min, 4 °C), washed three times with TGEM1 buffer (NaCl 1 M, Tris–HCl (pH 7.9) 20 mM, glycerol 20%, EDTA 1 mM, magnesium chloride 5 mM, NP-40 0.1%) followed by washes with TGEM0.1 buffer (as TGEM1, except NaCl 0.1 M). Beads were kept at 4 °C as 50% v/v suspension in TGEM0.1 until the experiment.

Proteasome α subunits were cloned into IVEC expression vector. ^{35}S-methionine labeled α subunits were prepared in TNT® T7 Coupled *Escherichia coli* S30 extract system (Promega, WI) according to manufacturer's specifications. For binding assays, protein loaded beads were blocked for 1 h in room temperature with 1% BSA, followed by 2 washes with TGEM0.1. Proteasomes or radiolabeled α subunits were incubated with the beads for 1 h at 4 °C and 1 h at room temperature. Beads were washed three times with TGEM0.1, boiled in reduced Laemmli sample buffer and analyzed by Immunoblotting. To detect proteasomes, the membranes were incubated with mouse anti-20S proteasome antibody (Calbiochem, MCP231, 1:15 000), followed by HRP-conjugated goat anti-mouse. Bound α subunits were detected by SDS–PAGE followed by autoradiography.

2.6. Characterization of XBP-1u degron

XBP-1^{CTu} was cloned in frame into pEGFP-C1. C-terminal truncations were generated by PCR. Vectors were transfected into 293T cell. About 24 h after transfection the cells were treated with ZL_3VS (25 μM) for up to 6 h. Equal amounts of cells were harvested at the indicated time points, lyzed in 1% SDS and proteins were loaded on SDS–PAGE. The amount of proteins was measured by immunoblotting to GFP using rabbit anti-GFP and to p97 as a loading control.

2.7. Fluorescence microscopy

About 48 h after transfection, 293T cells were treated or not with ZL_3VS (25 μM) for 6 h. Cells were washed in PBS and fixed in 2% PFA for 10 min at room temperature. After washing with PBS the cells were permeabilized with 0.25% triton X-100 (7 min, room temperature), followed by a block with 2% BSA. Cells were stained with anti-vimentin antibody (clone V9, Golden Bridge International, WI). Alexa-647 conjugated goat anti-mouse antibody (Invitrogen, CA) was used as a secondary antibody. Nuclei were visualized by Hoechst 33342 staining (0.4 μg/mL, 15 min, RT, Invitrogen, CA). Images were taken by confocal fluorescence microscope (Zeiss 410 microscope (PlanApochromat ×40)) using FluView 1000 v.1.5 software.

3. Results

3.1. XBP-1u undergoes direct proteasomal degradation

XBP-1u and XBP-1^{NT}were tagged with 6XHis and expressed in *E. coli*. The purified proteins were incubated with 20S or 26S