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Research Article

Destabilization of the VCP-Ufd1-Npl4 complex is associated with decreased levels of ERAD substrates

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Abbreviations:
ERAD, ER-associated degradation
RNAi, RNA interference
UPR, unfolded protein response
UFD, ubiquitin fusion degradation
UPS, ubiquitin–proteasome system
VCP, valosin-containing protein

ABSTRACT

p97/VCP associated with Ufd1-Npl4 is considered a key player in ER-associated degradation (ERAD). RNA interference (RNAi) of one component of the Ufd1-Npl4 heterodimer destabilizes the VCP-Ufd1-Npl4 complex inducing proteasome-dependent degradation of the other component and releasing free VCP. In contrast to RNAi of VCP, RNAi of Ufd1 or Npl4 depleting ~90% of the VCP-Ufd1-Npl4 complexes does not induce unfolded protein response, indicating that the Ufd1-Npl4 dimer is not involved in the regulation of ER function by VCP. RNAi of Ufd1 or Npl4 is associated with a 2-fold increase in the levels of polyubiquitinated proteins, which form dispersed aggregates often associated with calnexin-positive structures. However, contrary to the effects of proteasome inhibition, RNAi of Ufd1 or Npl4 does not induce an accumulation of α-TCR and δ-CD3, two ERAD substrates overexpressed in HeLa cells. Instead, a 60–70% decrease in their levels is observed. The decrease in α-TCR levels is associated with a 50% decrease of its half-life. Upregulation of the putative channel forming protein, derlin-1, may contribute to the increased degradation of ERAD substrates. To explain our findings, we propose a model, where association of emerging ERAD substrates with VCP-Ufd1-Npl4 is not required for their degradation but has a regulatory role.

Introduction

The ubiquitin–proteasome system (UPS) is responsible for the degradation of most proteins in eukaryotic cells [1]. Components of the UPS are found in the nucleus and in the cytosol but are excluded from the lumen of the endoplasmic reticulum (ER) [2]. Nevertheless, many ER proteins are degraded by the UPS via a process termed endoplasmic

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reticulum-associated degradation (ERAD) [3–10]. ERAD is a pathway for disposal of proteins which failed to pass the quality control mechanisms in the ER [11] as well as for the regulated degradation of normal resident ER proteins, such as IP3 receptors or HMG-CoA reductase [4,12–17]. ERAD substrates pose a topological challenge to the UPS because they must be retrotranslocated across and extracted from the ER membrane before and/or during their ubiquitination and subsequent proteasomal degradation. Retrotranslocation takes place either through the same Sec61 channel which serves for translocation of newly synthesized proteins into the ER [18,19] or through a different channel composed of derlin-1 and associated proteins [20,21].

ERAD requires the participation of multiple proteins both in the ER and in the cytosol [6,8,22–24] including the VCP-Ufd1-Npl4 complex in yeast as well as in mammalian cells [15,24–29], which also participates in the activation and extraction of processed transcription factors from multimeric complexes [27,30], and in the degradation of oligoubiquitinated substrates of the UPS [31]. A general model for the role of VCP-Ufd1-Npl4 involves a dual recognition and binding to both the polyubiquitin chain and the non-ubiquitinated portions of the emerging polypeptide at the cytoplasmic face of the ER membrane followed by substrate extraction/translocation and transfer to the proteasome [24,32]. In yeast, the requirement for the VCP-Ufd1-Npl4 complex has been shown for multiple substrates [22], while in mammalian cells, it is needed for the degradation of MHC class I heavy chains in permeabilized cells [20,21,24].

Polyubiquitin is bound by each of the individual components of the VCP-Ufd1-Npl4 complex [29]. VCP (valosin-containing protein, p97 or Cdc48 in yeast) is an abundant and ubiquitous hexameric type II AAA ATPase interacting with over 30 different proteins in the cytosol and nucleus [33–36], including the Ufd1-Npl4 heterodimer [26]. Ufd1 and Npl4 yeast mutants show an accumulation of ERAD substrates and defects in the processing of membrane-tethered transcription factors [15,25,27,28,30,37].

ERAD is a component of a coordinated cellular response to ER stress, termed the unfolded protein response (UPR) [7,38–40]. UPR is caused by the buildup of unfolded proteins in the ER and constitutes a mechanism to reduce this burden. In mammals, the UPR includes several distinct pathways that result in acute translational repression to reduce the burden of new proteins, increased expression of chaperones to aid folding of existing proteins, enhanced elimination of proteins that cannot be refolded, and apoptosis if ER stress is not relieved. A critical UPR pathway is initiated by activation of IRE-1, an ER membrane endonuclease that splices XBP-1 mRNA [41]. Translation of spliced XBP-1 mRNA produces a strong transcriptional activator, which activates the transcription of genes, whose products cope with the ER stress, including various genes coding for components of the ERAD machinery [42,43].

We have previously shown that RNA interference (RNAi) of VCP in HeLa cells results in the formation of large intracellular vacuoles derived from the ER, followed by apoptosis [44]. RNAi of VCP caused a generalized increase in polyubiquitinated proteins, indicative of impaired UPS function. Moreover, knockdown of Ufd1 and Npl4 also induced a discrete accumulation of polyubiquitinated proteins, while it failed to induce other phenotypic changes associated with VCP knockdown [44]. Since VCP is very abundant and it interacts with multiple proteins participating in different cellular functions [33–35], knockdown of VCP affects multiple pathways in addition to the pathway dependent on the VCP-Ufd1-Npl4 complex. To avoid those pleiotropic effects which may interfere with ERAD phenotype, we decided to study the role of this complex using RNAi to deplete the Ufd1-Npl4 dimer rather than VCP itself. In the present paper, we present evidence that allows as to hypothesize that mammalian VCP-Ufd1-Npl4 complex has a mostly regulatory role in ERAD; it may actually slow down the degradation of quality control substrates such as α-TCR and δ-CD3.

Materials and methods

Antibodies, reagents and plasmids

Anti-actin rabbit polyclonal antiserum was from Sigma (St. Louis, MO), anti-HA mouse monoclonal antibody was from Covance (Princeton, NJ), anti-ubiquitin mouse monoclonal antibody was from Santa Cruz (Santa Cruz, CA), anti-polyubiquitin FK1 mouse monoclonal antibody was from Biomol (Plymouth Meeting, PA), anti-TGN46 sheep polyclonal antiserum was from Serotec (Raleigh, NC) anti-VCP mouse monoclonal antibody was from BD Transduction Laboratories (Franklin Lakes, NJ), anti-GFP mouse monoclonal antibody was from Roche (Alameda, CA), while anti-BiP and anti-calnexin polyclonal rabbit antisera were from Stressgen (Victoria, Canada). p47 rabbit antiserum was a generous gift of Dr. Graham Warren (Yale University), anti-Rpt2 serum was a generous gift of Dr. George N. DeMartino (UT Southwestern) while anti-derlin-1 antibody was a generous gift of Dr. Tom Rapoport (Harvard University). Alkaline phosphatase-conjugated anti-mouse and anti-rabbit antibodies were from Promega (Madison, WI). Polyclonal sera against human Ufd1, Npl4 and derlin-1 proteins were custom-raised in rabbits after immunization with respective N-terminal peptides by Genemed Synthesis (San Francisco, CA). The plasmid encoding Ub-R-GFP (a gift from Dr. Maria Massucci, Karolinska Institute, Sweden) was derived from pEGFP-N1 [45]. The plasmids encoding HA-tagged α-TCR (gift of Dr. Ron Kopito, Stanford University) [46] and δ-CD3 (gift of Dr. Allan Weissmann, National Cancer Institute) [47] were derived from pcDNA 3.1. Unless otherwise stated, all the remaining reagents were from Sigma (St. Louis, MO).

Cell culture and establishment of stable cell lines

HeLa cells were grown in Advanced DMEM (Invitrogen, Carlsbad, CA) supplemented with Gluta-MAX™, antibiotic/antimycotic solution and 2% fetal bovine serum (Gemini Bioproducts, Woodland, CA). Plasmids used for transfection were sequenced at a core facility using CEQ 2000XL DNA Analysis System (Beckman Coulter, Fullerton, CA). Transfection was carried on using Lipofectamine 2000™ according to manufacturer's instructions (Invtrogen, Carlsbad, CA).