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Prolonged endoplasmic reticulum stress promotes mislocalization of immunoglobulins to the cytoplasm

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ABSTRACT

Signal peptide-dependent insertion of newly synthesized proteins into the endoplasmic reticulum (ER) is a multi-step process, whose fidelity varies with the identity of the protein and the cell type. ER translocation of prions is sensitive to conditions of acute ER stress in a manner that pre-emptively prevents their aggregation and proteo-toxicity. While this has been documented for extreme ER stress conditions and for a special type of proteins, the impact of chronic ER stress on protein translocation in general has not been well characterized.

The unfolded protein response (UPR) is a cytoprotective signaling pathway activated by ER stress. The transcription factor X-box-binding protein 1 (XBP-1) is a key element of the mammalian UPR, which is activated in response to ER stress. Deletion of XBP-1 generates constitutive chronic ER stress conditions. Chronic ER stress can also be produced pharmacologically, for example by prolonged treatment with proteasome inhibitors, which abrogates XBP-1 activation.

We tested the impact of chronic ER stress on protein insertion into the ER with special emphasis on antibody secreting cells (ASCs), as these cells cope physiologically with prolonged stress conditions. We show that XBP-1 in plasmablasts and fibroblasts controls the ER translocation of US2, a viral-encoded protein with a priori poor insertion efficiency. Using monoclonal antibodies that preferentially recognize ER-mis-inserted μ Ig chains we demonstrate that prolonged treatment of plasmablasts with proteasome inhibitors, as well as deletion of XBP-1, impaired the translocation of μ chains to the ER. Our data suggest that ASCs under prolonged ER stress conditions endure cytoplasmic mislocalization of Ig proteins. This mislocalization may further explain the exquisite sensitivity of multiple myeloma to proteasome inhibitors.

1. Introduction

In mammalian cells, it is estimated that 30% of all proteins are targeted to the endoplasmic reticulum (ER), the port of entry into the secretory pathway. The influx of newly synthesized polypeptide chains into the ER is variable, and can change rapidly in response to environmental signals, infection by pathogens or differentiation programs (Gass et al., 2002; He, 2006; Iwakoshi et al., 2003; Lee et al., 2005; Ozcan et al., 2004). ER stress occurs when cellular protein synthesis demand exceeds its folding capacity. In an effort to maintain homeostasis, a network of signaling cascades known as the unfolded protein response (UPR) is activated, resulting in reduced protein synthesis, enhancement of the ER folding capacity, increased degradation of ER misfolded proteins and enhanced ER biogenesis (Ron and Walter, 2007; Sriburi et al., 2004, 2007).

In the mammalian UPR three ER transmembrane sensors cooperate to attenuate the ER load: activating transcription factor 6 (ATF6), inositol requiring enzyme 1 (IRE-1) and PKR-like ER kinase (PERK). The first two pathways increase the folding capacity of the cell by upregulation of ER resident chaperones, expand the ER and increase the clearance of misfolded proteins through ER-associated degradation pathways, while the PERK pathway transiently inhibits protein translation (Ron and Walter, 2007). Continued activation of

Abbreviations: ASCs, antibody secreting cells; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GS, glycosylation site; HCMV, human cytomegalovirus; IP, immunoprecipitation; IRE-1, inositol requiring enzyme 1; PERK, PKR-like ER kinase; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response; XBP-1, X-box-binding protein 1.

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UPR due to lasting stress conditions will eventually lead to apoptosis (Hetz et al., 2006; Kim et al., 2008).

Protein translocation into the ER, as governed by a signal leader sequence, occurs co-translationally via the Sec61 translocon (Blobel and Dobberstein, 1975; Johnson and van Waes, 1999). The efficiency of translocation is not absolute and may vary substantially according to the cell type and the leader sequence in use (Levine et al., 2005). For some proteins, such as for prions and the human cytomegalovirus (HCMV)-encoded glycoprotein US2, the translocation efficiency into the ER is particularly low, and a substantial fraction thereof is translated directly in the cytoplasm (Gewurz et al., 2002; Rane et al., 2004). Interestingly, when prions translocate into a stressed ER they aggregate and exert toxic effects. Therefore, the enhanced mislocalization of prions to the cytoplasm under ER stress conditions is considered a pre-emptive measure against the proteo-toxicity associated with prion misfolding in the ER (Kang et al., 2006). However, excessive mislocalization may also lead to toxicity (Rane et al., 2004).

One of the main elements of the mammalian UPR is the transcription factor X-box-binding protein 1 (XBP-1). XBP-1 is activated by IRE-1-mediated non-canonical mRNA splicing in response to ER stress. The spliced form of XBP-1 (XBP-1s) is a potent transcription factor, whose targets encode ER chaperones, elements of the ER-associated degradation (ERAD) machinery and promote ER biogenesis (Calfon et al., 2002; Yoshida et al., 2001). In fact, XBP-1 is responsible for the induction of most UPR target genes (Shaffer et al., 2004; Shen et al., 2005), and prevention of its mRNA splicing by pharmacological or genetic means causes chronic ER stress conditions (Lee et al., 2003b).

XBP-1 has also been shown to play a pivotal role in the generation of cells that secrete excessive amounts of protein, such as plasma cells (Reimold et al., 2001). Immunoglobulin (Ig) production and secretion by these terminally differentiated B cells, inevitably requires adequate protein folding, brought about by efficient mechanisms coping with ER stress (Iwakoshi et al., 2003).

While protein mislocalization was overtly observed under severe ER stress conditions, inflicted by agents that globally perturb protein folding in the ER, such as thapsigargin (Tg) or tunicamycin (Tm) (Kang et al., 2006; Orsi et al., 2006), the effect of chronic ER stress on the efficiency of ER targeting has not been thoroughly studied. In this regard the specific role of XBP-1 in modulating protein targeting into the ER is elusive.

Here we show that deletion of XBP-1 in fibroblasts and in primary LPS-activated B cells promoted the mislocalization of US2. Overexpression of XBP-1s rescued the phenotype. To explore whether lack of XBP-1 splicing also impairs the ER translocation of proteins with high ER-targeting efficiency, we generated monoclonal antibodies that recognize with high efficiency mislocalized cytoplasm-deposited IgM μ heavy chains. We show that inhibition of XBP-1 splicing by prolonged treatment with proteasome inhibitors (Lee et al., 2003a), caused mistargeting of μ chains to the cytoplasm in a manner exacerbated by lack of XBP-1. Our data suggest that treatment of antibody secreting cells with proteasome inhibitors may promote global ER mislocalization of Ig molecules as well as proteins with low ER-targeting efficiency. This may provide further insight to the reason for the exquisite sensitivity of multiple myeloma to proteasome inhibitors in the clinics.

2. Materials and methods

2.1. Cell culture

Cell culture: wt and XBP-1$^{-/-}$ mouse fibroblasts were previously described (Lee et al., 2003b). 293T HEK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C in a 5% CO_2 incubator.

The hybridoma lines D2 and C2 (Nelson et al., 1983) and primary B cells were maintained in RPMI medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 unit/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH = 7.4), 1% non-essential amino acids, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Cells were treated with 2.5 μg/ml tunicamycin, 2.5 μg/ml thapsigargin or 10 μM of the proteasome inhibitor MG132 where indicated.

2.2. Isolation of primary B cells

Purified mature B cells were isolated from spleen of adult (6–10 weeks old) XBP-1$^{f/f}$ (wt) or CD19-CRExXBP-1$^{f/f}$ (XBP-1$^{-/-}$) mice by magnetic CD43 depletion bead selection according to the manufacturer's guidelines (Miltenyi Biotec, USA).

2.3. Cell transduction

293T cells were transfected by standard calcium phosphate precipitation method. MEFs were transfected by Effectene (QIAGEN GmbH, Germany) according to the manufacturer's protocol. Primary B cells were infected by retroviruses as previously described (Tirosh et al., 2005a). pcDNA3/US2 and pcDNA3/US11 were provided by Dr. Hidde Ploegh (WIBR, Cambridge, MA). pFLAG-XBP-1s was provided by Dr. Davis Ron (NYU, NY, NY). Retroviruses encoding US2 were generated in 293T using a triple transfection protocol with pMig/US2 and vectors encoding gagpol and Env, as previously described (Tirosh et al., 2005a).

2.4. Western blot analysis

Total cell lysates were prepared in 1% SDS and protein concentration was measured using a BCA method (Thermo, USA). Samples of equal protein content were separated by SDS-PAGE (12% acrylamide) and electro-transferred onto PVDF membranes. Rabbit polyclonal antibodies against p97, US2, US11, Sec61β, ERO1α and PDI were kindly provided by Dr. Hidde Ploegh (WIBR, Cambridge, MA). Antibody against Bip was purchased from Abcam. Antibody against β-actin and anti-FLAG mAb (M2) were purchased from Sigma. Goat anti-IgM was purchased from Southern Biotechnology Associates. HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

2.5. RNA isolation and RT-PCR

Total RNA from cells was isolated using TriReagent (Bio-lab, Israel) according to the manufacturer's protocol. cDNA was generated using RevertAid^{TM} first strand cDNA synthesis kit (from 2 μg of RNA with random hexameric primers), according to manufacturer's instructions. Primer sequences for XBP-1 splicing: Human XBP-1: Forward: 5′-CCTGGTTGCTGAAGAGGA-3′; Reverse: 5′-CCATGGGGAGATGTTCTG-3′; Mouse XBP-1: Forward: 5′-ACACGCTTGGGAATGGACAC-3′; Reverse: 5′-CCATGGGAAGATGTTCTGGG-3′. PCR conditions were described before (Tirosh et al., 2005a).

2.6. Radioactive pulse-labeling and immunoprecipitation (IP)

Cells were harvested, washed in PBS and resuspended in DMEM lacking methionine and cystein (starvation medium), and incubated for 45 min at 37 °C (starvation period). Unless specifically indicated, 25 μM of MG132 was included in the last 15 min of the starvation period. [^{35}S]-Methionine/cysteine mixture (PerkinElmer, NEG-072) was added in a ratio of 1:20 (v/v) for the indicated time. Cells were lysed in 1% SDS, and diluted in