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Decreased ER-associated degradation of α-TCR induced by Grp78 depletion with the SubAB cytotoxin

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ABSTRACT

HeLa cells stably expressing the α chain of T-cell receptor (αTCR), a model substrate of ER-associated degradation (ERAD), were used to analyze the effects of BiP/Grp78 depletion by the SubAB cytotoxin. SubAB induced XBP1 splicing, followed by JNK phosphorylation, eIF2α phosphorylation, upregulation of ATF3/4 and partial ATF6 cleavage. Other markers of ER stress, including elements of ERAD pathway, as well as markers of cytoplasmic stress, were not induced. SubAB treatment decreased absolute levels of αTCR, which was caused by inhibition of protein synthesis. At the same time, the half-life of αTCR was extended almost fourfold from 70 min to 210 min, suggesting that BiP normally facilitates ERAD. Depletion of p97/VCP partially rescued SubAB-induced depletion of αTCR, confirming the role of VCP in ERAD of αTCR. It therefore appears that ERAD of αTCR is driven by at least two different ATP-ase systems located at two sides of the ER membrane, BiP located on the lumenal side, while p97/VCP on the cytoplasmic side. While SubAB altered cell morphology by inducing cytoplasm vacuolization and accumulation of lipid droplets, caspase activation was partial and subsided after prolonged incubation. Expression of CHOP/GADD153 occurred only after prolonged incubation and was not associated with apoptosis.

1. Introduction

The endoplasmic reticulum (ER) comprises about half of the total membrane area and one-third of the newly translated proteins in a typical eukaryotic cell (Boyce and Yuan, 2006; Voeltz et al., 2002). After co-translational insertion into the ER, new proteins undergo folding, assembly, and posttranslational modifications which are scrutinized by a rigorous quality control mechanism (Ellgaard and Helenius, 2003). Misfolded proteins which fail to refold properly are retrotranslocated to the cytosol where they undergo degradation mediated by the ubiquitin and proteasome system (UPS), a process known as ER-associated degradation (ERAD) (Ahner and Brodsky, 2004; Ellgaard and Helenius, 2003; Kostova and Wolf, 2003; Sitia and Braakman, 2003; Tsai et al., 2002). An increase in protein misfolding within the ER leads to an integrated cellular response, which involves translational attenuation, decreasing the input of new proteins, followed by a transcriptional reaction known as unfolded protein response (UPR) (Hampton, 2003; Harding et al., 2002;

Abbreviations: CHX, cycloheximide; ER, endoplasmic reticulum; ERAD, ER-associated degradation; HUS, hemolytic-uremic syndrome; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCR, T-cell receptor; UFD, ubiquitin-fusion degradation; UPS, ubiquitin–proteasome system; VCP, valosin-containing protein.

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Ma and Hendershot, 2002; Shen et al., 2004). UPR leads to the upregulation of multiple proteins, including components of ERAD, which counteract at different levels the ER dysfunction caused by protein misfolding. Thus, UPR is an adaptative mechanism which promotes survival. However, prolonged UPR activation eventually triggers apoptosis (Boyce and Yuan, 2006; Wu and Kaufman, 2006).

A widely accepted and elegant model proposes that when the load of misfolded proteins in the ER exceeds the buffering capacity of the ER chaperone BiP – a condition known as ER stress – UPR is triggered through the depletion of free BiP (Boyce and Yuan, 2006; Hampton, 2003; Harding et al., 2002; Ma and Hendershot, 2002; Shen et al., 2004; Wu and Kaufman, 2006). BiP (Grp78) is a highly conserved and abundant ER chaperone of the Hsp70 family, comprising an N-terminal ATP-ase and a C-terminal protein binding domain, that is essential for the survival of eukaryotic cells (Lee, 2005; Luo et al., 2006). Under normal conditions, BiP binds to lumenal domains of three different transmembrane ER proteins: ATF6, IRE1, and PEK/PERK. During ER stress, BiP preferentially binds to misfolded proteins within the ER. Therefore, BiP detaches from the lumenal domains of those proteins, which now interact, oligomerize and trigger signaling events leading to UPR. Active PERK phosphorylates eIF2α, leading to a generalized repression of translation (Harding et al., 1999; Shi et al., 1998) associated with a selective translation of mRNAs bearing upstream open reading frames such as ATF3 and ATF4 (Shen et al., 2004). Active IRE1 mediates a unique cytosolic splicing of the mRNA coding for the XBP1 transcription factor (Yoshida et al., 2001) as well as generalized degradation of ER-associated mRNAs (Hollien and Weissman, 2006). Degradation of ER-associated mRNAs is accompanied by the co-translational degradation of nascent polypeptide chains (Oyadomari et al., 2006). At the same time ATF6 traffics to the Golgi, where it is cleaved by Site 1 and Site 2 proteases, leading to the release of a free cytosolic portion of ATF6, which translocates to the nucleus (Haze et al., 1999; Yoshida et al., 1998). XBP1, ATF6, ATF3 and ATF4 all induce a concerted transcription of multiple genes coding various ER chaperones, components of ERAD, and elements of the secretory pathway (Ng et al., 2000; Travers et al., 2000). Thus, UPR is an adaptative response allowing the cell to cope with ER stress. However, when UPR is persistent, it leads to apoptosis via transcriptional induction of CHOP/GADD153, TRAF-mediated activation of cJUN N-terminal kinase, and/or the activation of caspase-12/4 (Boyce and Yuan, 2006; Wu and Kaufman, 2006).

ERAD constitutively counteracts ER stress, eliminating misfolded proteins from the ER, but it is further activated as part of UPR (Casagrande et al., 2000; Friedlander et al., 2000). Expression of individual T-cell receptor (TCR) subunits in cell lines which endogenously do not express TCR have served in numerous studies to resolve the molecular determinants for rapid degradation of free TCR subunits (Bonifacino et al., 1989, 1990; Fang et al., 2001; Fayadat and Kopito, 2003; Huppa and Ploegh, 1997; Lenk et al., 2002; Tiwari and Weissman, 2001; Travers et al., 2000; Wileman et al., 1990, 1993; Yang et al., 1998; Yu et al., 1997; Yu and Kopito, 1999). αTCR is a type I transmembrane protein with a short cytoplasmic tail of 5 amino acids, which is dislocated from the ER, deglycosylated and degraded in the cytosol by 26S proteasomes (Huppa and Ploegh, 1997; Yang et al., 1998; Yu et al., 1997). Ubiquitination of αTCR is required not only for its targeting to the 26S proteasomes, but also for its retrotranslocation from the ER (Thrower et al., 2000; Tiwari and Weissman, 2001).

The AB_5 subtilase cytotoxin (SubAB) is produced by Shiga toxigenic strains of *Escherichia coli* (STEC) capable of inducing life-threatening complications of gastrointestinal disease, such as the hemolytic-uremic syndrome (HUS) (Paton et al., 2004). The name subtilase cytotoxin emanates from the similarity of the 35 kDa subunit A to members of the Subtilase S8 family of serine proteases, and is most similar to a protease produced by *Bacillus anthracis*; the B subunit of SubAB mediates binding of toxin to the surface of eukaryotic cells and bears similarity with putative exported proteins from *Salmonella typhi* and *Yersinia pestis* (Paton et al., 2004). SubB also directs internalization of the toxin and retrograde trafficking to the ER in a clathrin-dependent fashion (Chong et al., 2007). HUS is a life-threatening complication of STEC infection characterized by microangipathic hemolytic anemia, thrombocytopenia and renal failure, which can be reproduced in experimental mice by injection of purified SubAB (Wang et al., 2007). The A subunit of SubAB specifically cleaves BiP depleting cellular stores of this ER chaperone within 20–30 min upon addition to cell culture, an activity which is completely abolished by the Ser272Ala mutation of the A subunit (Paton et al., 2006). An independent group confirmed cytotoxic activity of SubAB, however it also has claimed an additional vacuolizing activity for the B subunit of SubAB (Morinaga et al., 2007).

SubAB induces features of ER stress in vivo and in vitro (Hayakawa et al., 2008; Paton et al., 2006), and has been recently shown to activate all three ER stress signaling pathways (IRE1, PERK and ATF6) in Vero cells (Wolfson et al., 2008). In the present study we have further explored the mechanisms of ER stress induced by SubAB in human cells, focusing in particular on its effects on the degradation of an established ERAD substrate such as αTCR.

2. Materials and methods

2.1. Antibodies, reagents and plasmids

SubAB and $SubA_{A272}B$ were purified by Ni-NTA chromatography as previously described (Paton et al., 2004, 2006; Talbot et al., 2005). Peak fractions were pooled, dialyzed against phosphate-buffered saline and stored in 50% glycerol at −20 °C, before addition to cell culture media at a final 1 μg/ml concentration (Paton et al., 2006). Anti-actin and anti-ubiquitin rabbit polyclonal antisera were from Sigma (St. Louis, MO), anti-HA11 mouse monoclonal antibody was from Covance (Princeton, NJ), anti-polyubiquitin FK1 mouse monoclonal antibody was from Biomol (Ply-