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

Agnieszka Lass^a, Marek Kujawa^b, Elizabeth McConnell^a, Adrienne W. Paton^c, James C. Paton^c, and Cezary Wójcik^{ad}

^aDepartment of Anatomy and Cell Biology, Indiana University School of Medicine – Evansville, Evansville, Indiana, USA

^bDepartment of Histology and Embryology, Center of Biostructure, Medical University of Warsaw, Warsaw, Poland, mkujawa@ib.amwaw.edu.pl

^cSchool of Molecular and Biomedical Science, University of Adelaide, South Australia, Australia, james.paton@adelaide.edu.au

Abstract

HeLa cells stably expressing the α chain of T-cell receptor (α TCR), a model substrate of ERAD (ER-associated degradation), were used to analyze the effects of BiP/Grp78 depletion by the SubAB cytotoxin. SubAB induced XBP1 splicing, followed by JNK phosphorylation, eIF2 α phosphorylation, upregulation of ATF3/4 and partial ATF6 cleavage. Other markers of ER stress, including elements of ER-associated degradation (ERAD) pathway, as well as markers of cytoplasmic stress, were not induced. SubAB treatment decreased absolute levels of α TCR, which was caused by inhibition of protein synthesis. At the same time, the half-life of α TCR 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 α TCR, confirming the role of VCP in ERAD of α TCR. It therefore appears that ERAD of α TCR is driven by at least two different ATPase systems located at two sides of the ER membrane, BiP located on the luminal side, while p97/VCP on the cytoplasmic side. While SubAB altered cell morphology by inducing cytoplasmic 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.

Keywords

T-cell receptor (TCR); endoplasmic reticulum (ER); ER-associated degradation (ERAD); unfolded protein response (UPR); subtilase; cytotoxin

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

to whom correspondence should be addressed at: Department of Anatomy and Cell Biology, Indiana University School of Medicine – Evansville, 8600 University Blvd., Evansville, IN 47712, USA Tel. 812-461-5437; Fax. 812-465-1184; e-mail: cwojck@iupui.edu.

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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; 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 luminal 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 luminal domains of those proteins, which now interact, oligomerize and trigger signaling events leading to UPR. Active PERK phosphorylates eIF2 α , 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 Site1 and Site2 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 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; Bonifacino et al., 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; Wileman et al., 1993; Yang et al., 1998; Yu et al., 1997; Yu and Kopito, 1999). α TCR 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 proteasomes (Huppa and Ploegh, 1997; Yang et al., 1998; Yu et al., 1997). Ubiquitination of α TCR 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 AB₅ 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 microangiopathic 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 α TCR.

Materials and Methods

Antibodies, reagents and plasmids

SubAB and SubA_{A272B} were purified by Ni-NTA chromatography as previously described (Paton et al., 2004; Paton et al., 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 $1\text{ }\mu\text{g/ml}$ 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 (Plymouth Meeting, PA), anti-HERP rabbit antiserum was from Abnova (Taipei, Taiwan), anti-eIF2 α , anti-CHOP/GADD152, anti-ATF3 and anti-ATF4 rabbit antisera were from Santa Cruz (Santa Cruz, CA), anti-ATF6 C-terminal and N-terminal rabbit polyclonal sera were from AnaSpec (San Jose, CA), anti-TGN46 sheep polyclonal antiserum was from Serotec (Raleigh, NC), anti-p97/VCP mouse monoclonal antibody was from BD Transduction Laboratories (Franklin Lakes, NJ), anti-PARP rabbit antiserum was from Roche (Alameda, CA), anti-Caspase 3 and anti-JNK rabbit antisera as well as anti-Hsp27 and anti-phospho-JNK mouse monoclonal antibodies were from Cell Signalling (Danvers, MA), anti-phospho-eIF2 α rabbit antiserum was from BioSource (Camarillo, CA), anti-calnexin rabbit antiserum as well as anti-Hsp70, anti-PDI and anti-KDEL mouse monoclonal antibodies were from Stressgen (Victoria, Canada). Cycloheximide (Sigma) was prepared as a 2.5 M stock solution in MiliQ water and used at final 50 mM concentration. MG132 (EMD Biosciences/Merck, Darmstadt, Germany) was prepared as a 10 mM stock solution in cell culture grade DMSO (Sigma) and used at a final 25 μM concentration. Thapsigargin and tunicamycin were prepared as 1 mM and 10 mg/ml stock solutions respectively, both in cell culture-grade DMSO (all from Sigma) and used at final concentrations of 1 μM and 10 Mg/ml respectively. Unless otherwise stated all the remaining reagents were from Sigma (St. Louis, MO).

Cell culture

HeLa cells, COS7 cells and HEK293 cell were acquired from ATTC (Manassas, VA). HeLa cells were grown in Advanced DMEM (Invitrogen, Carlsbad, CA) supplemented with 2% FCS (Gemini Bioproducts, Woodland, CA), HEK293 cells were grown in DMEM/F12 (Invitrogen) with 10% FCS (Gemini), while COS7 cells were grown in DMEM (Invitrogen) supplemented with 10% FCS (Gemini). All media were supplemented with Gluta-MAX™ and antibiotic/antimycotic solution, while media used for HeLa cells stably transfected with HA-tagged α TCR (Wojcik et al., 2006) were additionally supplemented with geneticin (all from Invitrogen).

RNA interference (RNAi)

Small interfering RNAs (siRNAs) were obtained from Dharmacon (Lafayette, CO). siRNAs targeting p97/VCP have been described previously (Wojcik et al., 2006). RNAi was performed using X-tremeGENE™ (Roche Applied Science, Penzberg, Germany) transfection reagent. Briefly, cells were seeded onto 6-well plates 24 h prior transfection, to reach 70% confluence on the day of transfection. 2.5 μ l of the siRNA stock in RNA-se free water was mixed with 3 μ l of the transfection reagent in 200 μ l of serum-free Advanced DMEM. After a 20 min preincubation this solution was added to 500 μ l of serum-free media to a final siRNA concentration of 20 nM. After 4 hrs 4 ml of full culture media was added without removal of the transfection mixture. Cells were collected 48 h after the transfection.

RT-PCR

RNA was isolated from cells collected in Trizol (Invitrogen, Carlsbad, CA) (Chomczynski and Sacchi, 1987) and semiquantitative RT-PCR was performed with the OneStep kit (Qiagen, Valencia, CA) using a pair of primers (IDT DNA, Coralville, IA) corresponding to appropriate nucleotides: 412–431 and 834–853 of XBP1 cDNA (which amplify the region including the 26 bp deletion dependent on IRE-1 endonuclease activity (Yoshida et al., 2001); 304–323 and 780–800 for BiP mRNA (X87949); 247–267 and 519–539 for derlin 1 mRNA (NM_024295); 177–197 and 499–519 for HERP mRNA (NM_014685); 75–95 and 301–320 for ATF3 mRNA (NM_004024); 378–398 and 730–750 for β 5 proteasome subunit mRNA (BC057840); and 792–811 and 1051–1070 for actin mRNA (NM_00110). DNA electrophoresis was performed on standard 1% agarose gels, DNA was labeled with ethidium bromide and images were acquired using Kodak Image Station MM (Eastman Kodak, Rochester, NY).

SDS-PAGE and Western blotting

Whole cell lysates were obtained in RIPA buffer (150 mM NaCl, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5 @ 25 °C) supplemented with Complete Mini™ protease inhibitors (Roche, Mannheim, Germany) and sodium orthovanadate. All samples were normalized to the same protein concentration determined using Bradford reagent (Biorad, Hercules, CA) (Bradford, 1976). Samples were resolved by standard SDS-PAGE using the MiniProtean II system (BioRad, Hercules, CA). Western blotting was performed on Immobilon-P PVDF membrane (Millipore, Billirica, MA). Primary antibodies were detected with secondary HRP-conjugated antibodies (Jackson Immunoresearch, West Grove, PA). HRP was detected using the Amersham ECL™ Advance kit (GE Healthcare, Piscataway, NJ). Images were acquired using Kodak Image Station 4000MM (Eastman Kodak, Rochester, NY). EZ-Run™ protein standards (Fisher Scientific, St. Louis, MO) have been used as molecular weight markers.

Determination of half-life

To calculate half-life, HeLa cells overexpressing α TCR were preincubated for 60 min (short time) or 15 h (long time) with either toxin before the addition of 50 mM cycloheximide. Cell

lysates were obtained at specified time points in RIPA buffer supplemented with protease inhibitors and sodium orthovanadate. Samples were resolved by SDS-PAGE and α TCR was detected with the anti-HA11 antibody. Densitometric analysis of the Western blots was performed using the Image Quant 5.2 software (Amersham Bioscience, Piscataway, NJ). The intensity of the individual bands was expressed as % remaining of the intensity at time point 0 and plotted on a semilogarithmic scale as a function of time. Curve fitting was performed using Microsoft Excel as an exponential function intersecting the ordinate at 100%. Squared R coefficients were calculated as a measure of fitting accuracy and are indicated on the graphs. Each experiment was repeated three times, degradation coefficients obtained for each experimental group (calculated for each experiment, $a = -\ln [\% \text{ of initial } \alpha\text{TCR remaining}] / [\text{time since } t=0]$) were compared with other group using Student's t-test.

Immunofluorescence microscopy

HeLa cells grown in Labtek two-chamber slides (Nunc Nalgene, Naperville, IL) were fixed in ice cold methanol. After fixation, cells were 3x washed with TBS, pH 7.6, supplemented with 0.1% bovine serum albumin and 0.1% fish gelatin (TBS+), and incubated with primary antibodies diluted in the same buffer containing Tween-20 for 2 hours. After three 15 minute washes in TBS+, the cells were incubated with secondary Cy2, Cy5 or TRITC-conjugated anti-rabbit, anti-sheep and anti-mouse F(ab')₂ fragments (Jackson Immunoresearch, West Grove, PA). DNA was stained by 5 min incubation in a 1 μ g/ml 4'-6-Diamidino-2-phenylindole (DAPI, EMD Biosciences) solution in TBS. After 3 washes in TBS, cells were mounted using Gel/Mount (Biomed, Foster City, CA). Slides were observed using the 60x Plan Apo objective of a Nikon Eclipse TE2000-U epifluorescence microscope. Images were acquired using the CoolSNAP ES CCD camera operated by the Metamorph 6.3 software (Fryer Company, Cincinnati, OH) and optically deconvoluted with the Autodeblur software (Media Cybernetics, Silver Spring, MD).

Transmission electron microscopy

HeLa cells were grown in 6 well plates and were submitted to a 6 h treatment with either SubAB or SubA_{A272B} before fixation in 2% glutaraldehyde in a cacodylate buffer, postfixed with OsO₄ in cacodylate buffer and then dehydrated with propylene oxide and embedded in PolyBed 812 (PolySciences Inc., Warrington, PA). Resin blocks were cut, mounted on Formvar carbon-coated grids and observed in a Jeol Jem 100S electron microscope (Tokyo, Japan).

MTT assay

The cytostatic/cytotoxic effects of SubAB were tested in a standard MTT assay as described elsewhere (Golab et al., 2000; Nowis et al., 2007; Wojcik et al., 1997). Briefly, HeLa cells were seeded onto a 96-well flat bottom microtiter plate (Flow Laboratories, VA) at a density of 1000 cells/300 μ l medium/well. Toxins were added to a final 1 μ g/ml concentration 24h after seeding and cells were collected at 24 h or 48 h later. MTT solution was added to each well for the last 3 hours of incubation. Cells were lysed using resuspension buffer containing SDS and isopropanol. The plates were read with ThermoMax microplate reader (Molecular Devices Corp., Union City, CA) using a 570 nm filter. Cell proliferation was measured by the increase in absorbance relative to the absorbance of untreated cells at time = 0 and expressed as % of initial signal. Relative growth = $(A_t - A_b) \times 100 / (A_0 - A_b)$, where A_b is the background absorbance, A_t is experimental absorbance at time = t, and A_0 is the absorbance at time = 0. Data were plotted on a semilogarithmic paper. Each experiment was repeated three times. Proliferation coefficients obtained for each experimental group were compared with other group using Student's t-test. All squared R coefficients were above 0.990.

Measurement of total protein synthesis

HeLa cells grown in 6-well plates were starved for 1 h in Met-deficient media, labeled for 30 min in Met-deficient media supplemented with 1 mCi/ml of ^{35}S Met (EasytagTM Express Protein Labeling Mix, PerkinElmer, Boston, MA) and either SubAB or SubA_{A272B}, washed and chased for various times in full media with either toxin. Cells were lysed directly in sample buffer, resolved by SDS-PAGE and blotted with actin antibody. Gels were Commassie stained and dried, then exposed to Super RXTM X-ray film (Fuji Photo Film, Tokyo, Japan). Densitometric analysis of the autoradiograms and Western blots was performed using the Image Quant 5.2 software (Amersham Bioscience, Piscataway, NJ). Experiments were repeated three times. Incorporation of ^{35}S -Met into newly synthesized proteins was adjusted for the amount of total proteins approximated by the levels of actin. Results obtained at individual time points were compared by Student's t-test between the two groups.

Results

SubAB induces ER stress in different cell lines

We have used two common epithelial cell lines to characterize the effects of SubAB on ER structure and function in human cells: cervical carcinoma HeLa cells and embryonic kidney HEK293 cells. Since cytotoxic effects of SubAB were first investigated on Vero cells derived from African green monkey, we have also included COS7 cells (Morinaga et al., 2007; Paton et al., 2004; Paton et al., 2006).

In order to assure a complete cleavage of BiP, we have used a 1 $\mu\text{g}/\text{ml}$ concentration of the SubAB toxin, which was well above the minimal concentration necessary to induce a complete cleavage of BiP in Vero cells within 1 h (Paton et al., 2006). After 6 h incubation it consistently induced the degradation of BiP in all three cell lines, while the inactive mutant SubA_{A272B} did not have any effect on BiP levels (Fig. 1A). Degradation of the full length BiP of ~72 kDa was accompanied by the appearance of the ~28 kDa BiP fragment in all cell lines as reported previously (Paton et al., 2006). Baseline expression of ER stress markers differed markedly among the three cell lines. For example, CHOP/GADD45 levels were high in HEK293 cells, low in HeLa cells and undetectable in COS7 cells. SubAB did not induce an increase in CHOP/GADD45 in any of the cell lines. While the levels of total eIF2 α were similar in all three cell lines, SubAB induced eIF2 α phosphorylation only in HEK293 cells. ATF4 was consistently induced in all three cell lines, while ATF3 was clearly induced only in HeLa cells. 6 h treatment with SubAB did not affect the levels of two additional ER chaperones, PDI and calnexin, as well as the levels of the ubiquitin-like protein HERP involved in ERAD and UPR. SubAB also did not affect the levels of two different cytoplasmic stress proteins, Hsp27 and Hsp70. The baseline expression of those chaperones was different in each cell line: COS7 had higher levels of PDI, HERP and Hsp27, while HEK293 cells had a high level of Hsp70, with undetectable Hsp27.

Since ER stress is usually associated with increased ER-associated degradation (ERAD) of proteins (Casagrande et al., 2000; Friedlander et al., 2000), we also looked at different components of the ubiquitin and proteasome system (UPS). The levels of polyubiquitinated proteins were similar in all three cell lines and were not affected by either wild type or mutant SubAB. Moreover, there were no changes in the levels of the $\alpha 7$ subunit of the 20S proteasome or in the Rpt2 and Rpt5 subunits of the PA700 proteasome activator (Fig. 1A).

Uncontrolled ER stress eventually leads to apoptosis (Boyce and Yuan, 2006). Since SubAB is cytotoxic to Vero cells (Morinaga et al., 2007; Paton et al., 2004; Paton et al., 2006) and leads to increased apoptosis in many organs following intraperitoneal injection in mice (Wang et al., 2007), we therefore explored whether SubAB induces apoptotic features in human cells. In

HeLa cells, SubAB induced activation of procaspase 3 as evidenced by cleavage of PARP and detection of trace amounts of cleaved caspase 3. However, in COS7 and HEK293 cells SubAB did not induce PARP cleavage. COS7 cells had endogenously activated caspase 3, which however was not enhanced by SubAB nor associated with PARP cleavage (Fig. 1A).

RT-PCR of XBP1 with primers overlapping the cytoplasmic splicing site demonstrated that a complete cytoplasmic splicing of the XBP1 mRNA was induced by SubAB in all three cell lines (Fig. 1B), consistent with findings in Vero cells (Wolfson et al., 2008). At the same time SubAB induced an increase in the expression of BiP, HERP and ATF3 mRNA in all three cell lines. While in HeLa and HEK293 cells the mutant, inactive SubA_{A272}B did not induce significant changes in the levels of ER stress markers, in COS7 cells it induced an incomplete XBP1 splicing as well as a small increase in the levels of BiP and HERP. The levels of mRNA of the β 5 subunit of the proteasome and those of derlin 1, an ER channel protein involved in ERAD, were not affected by SubAB in any of the cell lines. Levels of the actin mRNA served as loading controls.

Evaluation of the different features of ER stress induced by SubAB in HeLa cells

Next, we investigated how ER stress develops over time after exposure of HeLa cells to SubAB. The choice of the latter cells allowed us to take advantage of a HeLa clone stably expressing the α chain of the TCR, which can be used as a convenient measure of ERAD (Nowis et al., 2006; Wojcik et al., 2006; Yang et al., 1998; Yu et al., 1997). SubAB completely cleaved BiP within 1 h leaving only the ~28 kDa BiP fragment, while SubA_{A272}B did not induce any trace of BiP proteolysis even after 16 h (Fig 2A). Full length BiP started to reappear after 16 h of incubation with SubAB, but total levels of BiP (full length + 28 kDa fragment) increased ~40% during the 16 h incubation with SubAB, which correlated with an increase in the levels of BiP mRNA (Fig. 2B). Grp94 (detected by the same anti-KDEL antibody as BiP) was also induced following a 16 h incubation with SubAB.

Levels of the ERAD substrate α TCR rapidly declined in cells treated with SubAB reaching the limits of detectability at 16 h, while SubA_{A272}B did not affect α TCR levels at all. Levels of polyubiquitinated proteins as well as levels of two different subunits of the 26S proteasome, β 5 and Rpt2, did not change upon SubAB treatment. There was also no change in the levels of the ubiquitin-like protein HERP involved in ERAD (Hori et al., 2004; Schulze et al., 2005).

SubAB induced phosphorylation of eIF2 α which peaked 3 h after addition of the toxin, followed by a decrease in eIF2 α phosphorylation after 16 h of incubation. Similar results were reported in Vero cells (Wolfson et al., 2008). At the same time we have observed a discrete decrease in the abundance of total eIF2 α , which by 16 h reached statistical significance. Peak phosphorylation of eIF2 α induced by SubAB coincided with marked transient phosphorylation of JNK/SAPK, which then decreased to baseline levels. This was followed by ATF4 expression, which appeared at 3 h and peaked at 16 h post addition of SubAB. ATF3 was induced later at 6 h of treatment and increased even further at 16 h of treatment. ATF6 α expression was not induced, as seen with the C-terminal antibody. However, a limited degree of cleavage of preexisting ATF6 α was induced by 6 h and persisted by 16 h of treatment with SubAB, as seen with the N-terminal antibody, specifically detecting the cleaved p50 fragment of ATF6 α . Baseline expression of CHOP/GADD34 was low and appeared to be induced late, by 16 h of treatment with SubAB. No changes in the levels of other ER chaperones such as PDI and calnexin as well as the cytoplasmic chaperone hsp27 were observed even with 16 h incubation with SubAB.

SubAB induced both caspase 3 cleavage and PARP cleavage at 3 h, which were also detectable at 6 h, but disappeared at 16 h post addition of SubAB (Fig. 2 A). The cell proliferation/cytotoxicity MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at

24 h and 48 h post addition of the toxins has revealed that a portion of HeLa cells submitted to the treatment with SubAB survives this episode of ER stress and continues to grow, albeit at a significantly slower pace than untreated cells or cells treated with the inactive SubA_{A272B} toxin (Fig. 2 C).

RT-PCR of XBP1 with primers overlapping the cytoplasmic splicing site demonstrated that SubAB induced partial splicing of XBP1 mRNA as early as 1 h after addition to the culture media. By 3 h post addition unspliced XBP1 mRNA was undetectable (Fig. 2B). Interestingly a complete XBP1 splicing was observed even 16 h post addition of SubAB. Semi-quantitative RT-PCR of BiP demonstrated induction of BiP mRNA as early as 1 h post addition of SubAB. Levels of the β -actin transcript remained the same all the time revealing the lack of effect of this toxin on overall levels of gene transcription.

SubAB represses global levels of protein synthesis

Since phosphorylation of eIF2 α is associated with a repression of protein synthesis (Brostrom et al., 1995), we decided to check how SubAB affects global rates of protein synthesis. Incorporation of ³⁵S-Met into newly synthesized proteins drops to ~5% of control levels 1 h after the addition of SubAB and returns to baseline levels 6 h after incubation with the toxin (Fig. 3 AB). Interestingly, 16 h after SubAB addition, protein synthesis rates increased to ~200% of control values. At the same time SubA_{A272B} did not have any effects on rates of protein synthesis.

SubAB decreases ER-associated degradation of α TCR

When expressed in the absence of other TCR subunits, α TCR is a short lived protein which undergoes rapid clearance by ERAD (Nowis et al., 2006; Yang et al., 1998; Yu et al., 1997; Yu and Kopito, 1999). The rapid disappearance of α TCR from cells treated with SubAB (Fig. 2A) suggested increased ERAD. We therefore measured the half life of α TCR in the presence of SubAB by chasing cells in the presence of the protein synthesis inhibitor, cycloheximide (CHX), and measuring α TCR levels at different time points of incubation with SubAB (Fig. 4 A,B). The $t_{1/2}$ of α TCR was not significantly affected by the treatment with the mutant control SubA_{A272B} toxin (80 min versus 70 min in untreated cells, $p=0.273$). Preincubation of cells for 60 min with SubAB significantly increased $t_{1/2}$ of α TCR as compared with control (120 min, $p=0.037$). We next wanted to evaluate whether this effect of SubAB on ERAD persists after a prolonged exposure of cells to SubAB. We therefore preincubated HeLa cells for 15 h before measuring the degradation rate of α TCR. Surprisingly, under these conditions the $t_{1/2}$ of α TCR was extended even further (210 min, $p=0.028$ from control, $p=0.013$ from the short incubation with SubAB). Incubation of HeLa cells for 3 h with the proteasome inhibitor MG132 induced an accumulation of α TCR, which was significantly less pronounced in the presence of CHX, indicating that much of the observed degradation involves newly synthesized protein.

RNAi of p97/VCP partially restores α TCR levels depleted by SubAB

In order to gain insights into the possible mechanisms of SubAB effects on α TCR levels, we combined treatment with SubAB with depletion of p97/VCP, an AAA ATP-ase involved in ERAD (Tsai et al., 2002; Ye et al., 2001; Ye et al., 2003). RNAi of p97/VCP with two different siRNAs induced an increase in the levels of glycosylated α TCR as previously published (Wojcik et al., 2006). A 16 h treatment with SubAB induced a decrease in α TCR levels, which was attenuated in cells submitted to RNAi of p97/VCP. Treatment with SubA_{A272B} did not affect α TCR levels at all regardless of the p97/VCP status (Fig. 4 C).

SubAB interferes with UPR induced by tunicamycin and thapsigargin

To further assess the mechanisms of action of this bacterial toxin, we evaluated the effects of SubAB on the induction of UPR by two classic ER stressors, tunicamycin and thapsigargin (Fig. 5). As expected, 16 h treatment with either tunicamycin or thapsigargin induced ER stress and subsequent UPR as evidenced by the induction of ATF3, ATF4, BiP and Grp94, accompanied by induction of XBP1 splicing. At the same time, SubAB also induced a complete XBP1 splicing, which was unaffected by thapsigargin or tunicamycin. SubAB degraded BiP regardless of the presence of tunicamycin and thapsigargin and induced the appearance of the ~28 kDa fragment. However, in the presence of SubAB, tunicamycin and thapsigargin failed to induce BiP expression as evidenced by the lack of increased levels of the ~28 kDa BiP fragment. Both tunicamycin and thapsigargin induced the expression of ATF3 and ATF4, which were also induced by SubAB treatment, however in SubAB treated cells, tunicamycin and thapsigargin were not able to further induce ATF3 and ATF4. No differences were noted in the levels of several proteins involved in ER and ERAD such as HERP, VCP or the $\alpha 7$ and Rpt2 proteasomal subunits. The mutant SubA_{A272B} toxin did not interfere in any way with the effects of tunicamycin or thapsigargin.

SubAB induces vacuolization of cytoplasm and accumulation of intracellular lipids

In order to assess any morphological changes in the structure of the ER or the Golgi which may be induced by SubAB, we treated HeLa cells for 6 h with either SubAB or Sub A_{272B} and processed them for immunofluorescence microscopy (Fig. 6 A,B) and transmission electron microscopy (Fig. 7). Immunofluorescence has revealed that >80% of cells treated with SubAB develop perinuclear vacuoles, whose content remains unlabeled, but whose margins show labeling with markers of the ER (calnexin and BiP) and late Golgi (TGN46) compartments as well as increased labeling with the FK2 antibody, specific for polyubiquitin chains. Similar vacuoles were very scarce among untreated cells (<2%) or cells treated with the control mutated toxin (<5%).

As observed by electron microscopy untreated cells have big, eccentric nuclei with clear nucleoli, rugged surface and moderately dense cytoplasm, with abundant SER and RER cisternae, Golgi stacks, mitochondria and lysosomes (Fig. 7 A). SubA_{A272B} did not affect most of the cells, which were not different from control (Fig. 7 B), while few cells did show a limited swelling of ER cisternae and dispersed vacuoles (not shown). In contrast, most cells treated with SubAB revealed marked ultrastructural changes (Fig. 7 C,E,D,F). Both cytoplasm and nuclei appeared swollen with formation of multiple vacuoles, derived from elements of the ER, Golgi and probably also mitochondria. There appeared to be a bimodal distribution of the vacuoles, since they predominated around the nucleus and at the cell periphery, being relatively scarce in between (Fig. 7 C). The nuclear envelope was usually also visibly swollen (Fig. 7 C,D). Typical RER structures were absent indicating dissociation of ribosomes from ER cisternae (Fig. 7 D). Lipid droplets were found in the cytoplasm of multiple cells (Fig. 7 F), which were bigger and more abundant after 16 h incubation with SubAB (not shown). Other cells appeared more electron dense than control cells, displaying features of peripheral chromatin condensation, characteristic of apoptosis (Fig. 7 E).

Discussion

It has been recently shown that in green monkey Vero cells SubAB activates all three branches of UPR (Wolfson et al., 2008). We now confirm those studies extending them to human cells. We also provide an insight into the mechanisms of induction of ER stress by the SubAB cytotoxin. Since SubAB may be associated with HUS pathogenesis, our study may therefore provide important cues for the development of new therapeutic strategies. HUS is the most common cause of acute renal failure in infants and young children, and is a substantial cause

of acute mortality and chronic morbidity, in particular in underdeveloped countries with poor sanitary conditions and inappropriate hospital care (Siegler and Oakes, 2005). Injection of purified SubAB into experimental mice induced their demise after development of symptoms resembling HUS pathology, raising the possibility that SubAB contributes to HUS pathogenesis in humans (Wang et al., 2007). A preexisting infection with human CMV, which induces ER stress, was shown to prevent the cytotoxic effects of SubAB in human fibroblasts (Buchkovich et al., 2008). We have now observed that SubAB leads to a long lasting decrease of ER-associated degradation (ERAD). Such adaptation may play an important role in the prosurvival response, since it spares existing ER proteins in the absence or decrease of new protein synthesis.

ERAD constitutively counteracts ER stress, eliminating misfolded proteins from the ER, however UPR induces its further activation through upregulation of its components (Casagrande et al., 2000; Friedlander et al., 2000). We therefore expected that treatment with SubAB would accelerate ERAD of α TCR. Following treatment with SubAB we observed a rapid decrease in α TCR levels. However, we did not observe an associated decrease of its half life. Instead, its half life increased 2-fold indicating a decrease in ERAD. Therefore, rapid decrease of α TCR levels upon treatment with SubAB must be explained by another mechanisms such as global inhibition of protein synthesis (Harding et al., 1999; Shi et al., 1998), which we have indeed confirmed directly. In untreated cells, α TCR probably accounts for a significant fraction of total ERAD, while under ER stress induced by BiP depletion, remaining α TCR must compete for the ERAD machinery with a plethora of endogenous misfolded proteins. Accumulation of α TCR in toxin treated cells which were incubated with MG132 indicates that most of it is still degraded by the UPS pathway.

Our hypothesis that the decline in α TCR levels represents an inhibition of its synthesis satisfactorily explains the observations at the beginning of SubAB treatment, when global protein synthesis is inhibited. However, following 16 h of exposure to SubAB the half life of α TCR was further extended, while global protein synthesis levels were increased to ~200% of the levels observed in untreated cells. Those apparently contradictory observations can be reconciled only if we assume that the so called “global protein synthesis” measured by a bulk of ^{35}S -Met incorporation in reality reflects the synthesis of ER chaperones and ER structural proteins, while the synthesis of other cellular proteins, lacking ERSE or UPR responsive sequences, is still turned off. Since α TCR has a CMV promoter and was introduced randomly into the genome during the creation of the stable cell line its expression is still repressed under those conditions. This agrees with the finding that treatment of human fibroblasts with SubAB during the early phase of CMV infection downregulates the expression of viral proteins even as late as 96 h after addition of the toxin (Buchkovich et al., 2008). Many reports have indicated that ERAD components are upregulated as part of UPR (Casagrande et al., 2000; Friedlander et al., 2000). However, we have not observed an upregulation of proteins involved in ERAD, such as proteasome subunits, p97/VCP or the channel forming protein derlin 1 even after 16 h of incubation with SubAB.

The extended half life of α TCR in the absence of BiP may also be explained by assuming an important role of BiP in retrotranslocation. Indeed, BiP has been implied as the ER chaperone which can provide the force “pushing out” a polypeptide chain from the ER lumen into the cytosol (Winkler et al., 2003). Another model has emphasized the role performed by the cytosolic ATP-ase p97/VCP, as the molecular motor providing a force which “pulls out” the retrotranslocating polypeptide to the cytosol (Tsai et al., 2002). We have previously shown that p97/VCP participates in the degradation of α TCR, since depletion of p97/VCP by means of RNA interference caused features of ER stress accompanied by increased levels of α TCR and an extension of its half-life (Wojcik et al., 2006). When we treated p97/VCP-depleted cells with SubAB for 16 h, we observed an increase in α TCR levels compared with the effect of the

toxin alone, demonstrating a further impairment of ERAD, which led to increased levels of α TCR. Our results therefore indicate that ERAD of α TCR can be best explained by combining the “push” and “pull” models together, with BiP facilitating the retrotranslocation on the luminal side and p97/VCP facilitating it on the cytoplasmic site of the ER membrane. Further experiments will be required to directly demonstrate the involvement of BiP in retrotranslocation, since it may facilitate ERAD at an earlier stage, e.g. targeting to the retrotranslocation complex.

SubAB induced a particular sequence of changes in HeLa cells. Concomitant with a complete degradation of BiP within the first hour of incubation we observed early XBP1 splicing, eIF2 α phosphorylation and an almost complete suppression of protein synthesis, while activation of the ATF6 branch of UPR was delayed up to 6 h after initiation of treatment with SubAB, and was incomplete even after 16 h of incubation. By three hours of incubation, there was a transient wave of JNK phosphorylation coinciding with a peak in eIF2 α phosphorylation and activation of caspases. By six hours, caspases were still active. However, we never observed a complete caspase splicing, as seen after the treatment of HeLa cells with the reducing agent DTT, a strong inducer of ER stress (results not shown). At the same time, ATF4 was induced while eIF2 α phosphorylation and global protein synthesis already returned to pre-treatment levels. Interestingly, electron microscopy has revealed that at this stage there is still no association of ribosomes with swollen ER cisternae, while after 16 h of treatment with SubAB rough ER is abundant (not shown). This observation suggests that at 6 h most of the “global protein synthesis” may reflect synthesis of cytoplasmic, not ER-associated proteins. By 16 h after addition of the toxin, some uncleaved BiP appeared to be detected, but most BiP still remained as the 28 kDa fragment, indicating persistent subtilase activity of the toxin. The 28 kDa BiP fragment appeared to be stable, but the exact mechanism of its clearance and/or interactions is unknown. Nevertheless, total levels of BiP were increased by 40% and BiP mRNA was induced in accordance with other reports (Hayakawa et al., 2008; Wolfson et al., 2008). This contrasts with the effect of SubAB on human fibroblasts, where even after 96 h of incubation there were no signs of uncleaved BiP and levels of the ~28 kDa fragment did not increase (Buchkovich et al., 2008). Moreover, at 16 h post addition of SubAB, XBP1 splicing was still complete, ATF4 expression was high and Grp94 levels were increased. At no single time point did SubAB induce any changes in the levels of cytoplasmic chaperones or elements of the ubiquitin-proteasome system. Other ER chaperones such as PDI or calnexin were also not induced suggesting great flexibility of mammalian cells in adapting to a complete depletion of BiP.

Caspase activity was no longer detected after 16 h of treatment with SubAB indicating a complete adaptation of surviving cells to continuing ER stress. Interestingly, this early wave of apoptosis was not associated with increased expression of CHOP/GADD153, a proapoptotic transcription factor considered to be a mediator of extended ER stress (Oyadomari et al., 2002). CHOP/GADD153 was not upregulated until 16 h of treatment, but by that time caspase activation was no longer detected. Moreover, using a standard MTT assay, we observed that even long incubation (up to 48 h) with SubAB does not prevent cell proliferation, but only slow sit down. Electron microscopy of HeLa cells following 16 h of incubation with SubAB revealed two populations of cells: one group included cells with extreme vacuolization, accumulation of lipid droplets, apoptosis or necrosis, while another group included roughly normal cells with minimal vacuolization (not shown). The first population represents the cells which succumbed to the initial insult, while the latter population likely represent the cells which have successfully mounted UPR and survived. Our results contrast with the effects of SubAB on experimental mice, where evidence of increased apoptosis was detected in different organs 24 and 48 h following intraperitoneal SubAB injection (Wang et al., 2007).

In accordance with other reports of the effects of SubAB on mammalian cells we observed vacuolization of HeLa cells after treatment with SubAB (Morinaga et al., 2007). Formation of intracellular vacuoles is a common response to some, but not all, stimuli causing ER stress in HeLa cells, such as RNAi of p97/VCP (Wojcik et al., 2006) or extended inhibition of ERAD by proteasome inhibitors (Wojcik et al., 1996). However, in contrast to the two latter conditions, vacuoles induced by SubAB appeared to be derived not only from the ER but also from elements of the Golgi and from mitochondria. We have also observed changes in electron density of treated cells, indicating either swelling of nuclei and cytoplasm, or on the contrary their overcondensation, associated with apoptotic features. Interestingly, such dramatic changes in the cytoplasm were not associated with induction of cytoplasmic chaperones. In contrast to Morinaga et al. who associated the vacuolating activity with the B subunit of SubAB (Morinaga et al., 2007), we have observed only minimal changes after treatment with the mutant toxin, which can be explained by trace residual catalytic activity of SubA_{A272B} (Wolfson et al., 2008). Therefore, we must conclude that formation of the perinuclear vacuoles is downstream of BiP degradation and not an independent activity. It may correspond to the formation of new ER and Golgi membranes and/or aberrant function of ER. Accumulation of lipid droplets observed by electron microscopy may represent an activation of synthesis of new lipids, which eventually are incorporated into the ER membranes. In contrast to previous observations in human fibroblasts treated for 12 h with SubAB, we did not observe a significant increase in the number of secondary lysosomes and autophagic vacuoles (Buchkovich et al., 2008). It appears that human fibroblasts failed to mount an appropriate UPR, since they did not increase the level of BiP expression following SubAB treatment. Unfortunately, we can not further elaborate the reasons for this difference since the authors did not investigate other markers of ER stress (Buchkovich et al., 2008).

Overall, we have characterized the effects of SubAB-mediated BiP depletion in human epithelial cells. SubAB-treated cells quickly activated UPR as evidenced by almost immediate XBP1 splicing, followed by JNK phosphorylation, ATF3/4 expression as well as upregulation of Grp94 and BiP. Interestingly, other markers of ER stress, including elements of ERAD, were not induced. Instead of inducing ERAD activation and upregulation as part of UPR, SubAB led to its long lasting inhibition, as evidenced by increased half life of α TCR substrate. Such finding point toward an important role for BiP in the retrotranslocation process as suggested by others (Winkeler et al., 2003). While SubAB altered cell morphology by inducing cytoplasm vacuolization, caspase activation was only partial and transient. Surviving cells were able to proliferate despite persistent activity of the toxin. Future experiments will look into details of the interplay between prosurvival and proapoptotic factors in SubAB treated cells.

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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Fig. 1.

Induction of UPR by SubAB in different cell lines. HeLa cells, COS7 cells or HEK293 cells have been treated for 6 hr with either the wild type SubAB or the mutated SubA_{A272B} at 1 µg/ml concentration before being harvested for SDS-PAGE and Western blotting (A) or semi-quantitative RT-PCR (B). Asterisks indicate non-specific bands detected with the anti-KDEL antibody, arrowheads indicate the cleaved forms of caspase 3 and PARP, while the arrows indicates the three different species detected with the anti-KDEL antibody (Grp94, full length BiP and BiP fragment) as well as the spliced form of XBP1. SubAB induces cleavage of BiP and several features of ER stress as indicated by upregulation of multiple markers at both the protein and mRNA level.

Fig. 2.

Time-response of HeLa cells to SubAB treatment. the HeLa cells stably expressing α TCR were submitted to the treatment with either the wild type SubAB or the mutated SubA_{A272}B at 1 μ g/ml concentration for the indicated times before being harvested for SDS-PAGE and Western blotting (A) or semi-quantitative RT-PCR (B). SubAB induces an immediate BiP degradation, associated with early ER stress and apoptotic features, while SubA_{A272}B did not induce ER stress, even after prolonged incubation. Arrowheads point cleaved fragments of caspase 3 and PARP, while (C) and (N) denote C-terminal and N-terminal ATF6 antibodies respectively; (C) Results of an MTT assay performed 24 h and 48 h after the addition of SubAB or SubA_{A272}B compared with untreated cells. Asterisk indicates statistical significance between SubAB and both untreated and SubA_{A272}B -treated cells as calculated by Student's T test.

Fig. 3.

Effects of SubAB treatment on global protein synthesis. HeLa cells were starved in Met-deficient media, labeled with ³⁵S-Met, -chased in media supplemented with either SubAB or SubA_{A272}B for the indicated times and processed for SDS-PAGE. (A) Autoradiograph showing the global levels of ³⁵S-Met-labeled proteins in HeLa cell lysates, Coomassie blue stained gel showing total protein levels in the lysates, and corresponding Western blot showing levels of actin in the lysates; (B) Rates of protein synthesis, calculated as % of ³⁵S-Met-labeled proteins incorporated in untreated cells, normalized for actin to account for different protein load. Results represent means of three different experiments. Asterisks indicate statistical difference (p<0.002) as calculated by Student's T test.

Fig. 4.

Effects of SubAB on ERAD of α TCR. Degradation of α TCR in cells treated either with the wild type SubAB or the SubA_{A272B} mutant toxin. (A) Western blots showing the levels of α TCR, BiP, ubiquitin and actin in whole cell lysates obtained after the treatment for the indicated times with 1 μ g/ml toxins, 25 μ M MG132 and 50 mM CHX; (B) Calculation of α TCR degradation rates in cells treated with either SubAB or SubA_{A272B} toxins. The $t_{1/2}$ of α TCR is not different from control in cells treated with the mutant control SubA_{A272B} toxin (80 min versus 70 min, $p=0.273$), while both the short and long incubation with SubAB significantly increased $t_{1/2}$ of α TCR compared with control (120 min and 210 min respectively, $p=0.037$ and 0.028 respectively) and versus each other ($p=0.013$). Values represent means from three independent experiments with vertical bars representing SD; (C) Western blots showing the effects of a combination of 48 h RNA of p97/VCP with a 16 h treatment with either the wild type SubAB or the SubA_{A272B}. SubAB-induced decrease in α TCR levels was less pronounced in cells deficient for p97/VCP.

Fig. 5. Combination of SubAB with tunicamycin and thapsigargin treatment. HeLa cells have been treated with either the wild type SubAB or the mutated SubA_{A272B} at 1 µg/ml concentration either alone or in combination with 10 µg/ml tunicamycin or 10 µM thapsigargin mM PBA before being harvested for SDS-PAGE and Western blotting at 16 h (A) or at 6 h for semi-quantitative RT-PCR (B). Asterisks indicate non-specific bands detected with the anti-KDEL antibody. SubAB interferes with induction of ATF4 and BiP by tunicamycin and thapsigargin.

Fig. 6 A

Fig. 6 B **Fig. 6.**

Morphological changes induced by SubAB treatment. HeLa cells were treated for 6 h with either the wild type SubAB or the mutated SubA_{A272B} at 1 µg/ml concentration. While in cells treated with SubA_{A272B} small round vacuoles are sometimes found in individual cells (arrow), SubAB induces the formation of multiple round vacuoles in most treated cells. (A) Immunofluorescence images using anti-calnexin antibody as a marker of ER, anti-TGN46 antibody as a marker of Golgi and anti-FK2 antibody detecting polyubiquitinated proteins, scale bar represents 10 µm; (B) Immunofluorescence images using anti-actin antibody as a marker of cytoskeleton, anti-KDEL antibody as a marker of ER and DAPI to stain cell nuclei, scale bar represents 10 µm.

Fig. 7.

Electron micrographs showing ultrastructural changes upon treatment of HeLa cells with wild type or mutant SubAB. (A) control cells and (B) cells treated with SubA_{A272}B show normal ultrastructure of nuclei and cytoplasm with abundant mitochondria, lysosomes, Golgi stacks and ER cisternae, which are shown at higher magnification in insets in order to highlight ribosomes attached to ER leaflets. Cells treated with SubAB show extensive vacuolization (C), apoptotic changes (E, arrows point to peripherally condensed chromatin) and accumulation of lipid droplets (F, arrow). A magnification of (C) shows swelling of the nuclear envelope (D, arrows). nu – nuclei, nl – nucleoli, v – intracytoplasmic vacuoles. White box on panel F corresponds to 500 nm.