Effect of the unfolded protein response on ER protein export: a potential new mechanism to relieve ER stress

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Abstract

The unfolded protein response (UPR) is an adaptive cellular response that aims to relieve endoplasmic reticulum (ER) stress via several mechanisms, including inhibition of protein synthesis and enhancement of protein folding and degradation. There is a controversy over the effect of the UPR on ER protein export. While some investigators suggested that ER export is inhibited during ER stress, others suggested the opposite. In this article, their conflicting studies are analyzed and compared in attempt to solve this controversy. The UPR appears indeed to enhance ER export, possibly via multiple mechanisms. However, another factor, which is the integrity of the folding machinery/environment inside ER, determines whether ER export will appear increased or decreased during experimentation. Also, different methods of stress induction appear to have different effects on ER export. Thus, improvement of ER export may represent a new mechanism by which the UPR alleviates ER stress. This may help researchers to understand how the UPR works inside cells and how to manipulate it to alter cell fate during stress, either to promote cell survival or death. This may open up new approaches for the treatment of ER stress-related diseases.

Keywords ER stress · ER export · Unfolded protein response · MAPK · Sec16 · COPII · ERK2 · ER exit sites

Introduction

The endoplasmic reticulum (ER) is the first compartment in the constitutive secretory pathway where newly synthesized proteins are folded and oligomerized with the aid of chaperones and folding enzymes (Vincenz-Donnelly and Hipp 2017). After correct folding, client proteins leave the ER mainly in COPII-coated vesicles and move to the Golgi for further modifications and sorting. These vesicles bud from the ER membrane at specific sites termed ER exit sites (ERES). The assembly of COPII coat at ERES (D'Arcangelo et al. 2013) starts with the activation of the GTPase Sar1 by the guanine nucleotide exchange factor Sec12. Activated Sar1 is then inserted into the ER membrane and recruits Sec23/24 to form the inner coat layer, which helps cargo loading in the assembling vesicles. Sec13/31 is then recruited to form the outer coat layer.

Accumulation of unfolded and misfolded proteins inside the ER causes ER stress. This in turn activates an adaptive response called the unfolded protein response (UPR) which

works to alleviate stress via several mechanisms, including inhibition of client protein influx into the ER, enhancement of protein folding, and enhancement of protein degradation (Grootjans et al. 2016; Vincenz-Donnelly and Hipp 2017). In mammals, the activation of the UPR is mediated by three ER transmembrane stress sensors: PKR-like ER kinase (PERK), inositol requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). Failure of the UPR to restore ER homeostasis leads to cell apoptosis.

Studies in different species have shown that the UPR upregulates several genes involved in ER export and ER-to-Golgi transport (Murray et al. 2004; Saito et al. 2009; Teske et al. 2011; Travers et al. 2000), suggesting that the UPR may enhance ER export. This seems reasonable, as the generation of correctly folded proteins inside the ER is expected to increase after the upregulation of the protein folding machinery by the UPR, and this will necessitate an improvement of ER export activity to efficiently evacuate these proteins from the ER.

Strikingly, some investigators found that the induction of ER stress was accompanied by signs of ER export inhibition. Others found that the induction of ER stress was accompanied by signs of ER export enhancement. In this paper, the results of these conflicting studies are analyzed and compared, and other related evidences are presented to attempt to solve this controversy.

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Conflicting studies

Studies supporting inhibition of ER export during ER stress

In one study, Amodio et al. (2009) showed that treatment of human hepatoma Huh7 cells with the ER stressor thapsigargin (TG) reduced anterograde traffic of a reporter glycoprotein from the ER to the Golgi without compromising its folding and oligomerization. TG treatment also inhibited the reformation of the ER-Golgi intermediate compartment and the cis-Golgi after their breakdown and redistribution into the ER by brefeldin A (BFA). Moreover, TG was shown to decrease the number of exit sites on the ER membrane.

In a second study, Amodio et al. (2013) showed that inducing ER stress in Huh7 cells using three different agents (TG, DTT, or MG132) reduced the amount of COPII components (Sar1a/b, Sec23a, and Sec31a) bound to intracellular membranes. In addition, Sec23a was shown to cycle faster at ERES after TG treatment, reflecting reduced membrane stability of this protein under such condition.

Studies supporting enhancement of ER export during ER stress

In one study, Farhan et al. (2008) showed that prolonged overexpression of a cargo protein (for 24 h) in HeLa cells induced ER stress and activated the UPR. It also increased Sec24 and Sec16 protein expression, increased Sec24 and Sec16 binding to ER membrane, and increased the size and number of ERES suggesting an enhancement of ER export. This increase in ERES number depended on the presence of Sec16, suggesting the involvement of this protein in the enhancement process (shown below). Furthermore, this increase in ERES number required the activation of the UPR (IRE1 pathway). Mouse embryonic fibroblast (MEF) cells lacking IRE1 could not increase ERES number in response to chronic cargo overload.

Analysis and comparison

Can misfolded proteins escape from the stressed ER?

In their studies, Amodio et al. found signs of ER export inhibition after triggering ER stress. The authors suggested that the stressed cell may inhibit ER export to hold the increased amount of misfolded proteins inside ER where they can be dealt with and to prevent them from escaping and filling up post-ER compartments and that this may help cell to overcome ER stress.

One might ask: will the accumulated misfolded proteins escape from the ER and fill post-ER compartments if ER export remains highly functional during stress? In other

words, is inhibition of export the main barrier that holds misfolded proteins inside the stressed ER?

It should first be noted that mammalian ER contains a highly developed quality control system (an early barrier before ER exit) that can efficiently hold unfolded and misfolded proteins inside ER while allowing only mature proteins to travel to the Golgi (Vincenz-Donnelly and Hipp 2017). Briefly, when newly synthesized proteins enter ER, they bind to ER resident proteins such as chaperones and folding enzymes which assist their folding. This association with resident proteins continues until complete folding or degradation. Also, this association can hold client proteins inside the ER and prevent their passage to the Golgi. After folding, client proteins dissociate from the folding machinery and get scanned by the folding sensors, which are resident proteins that can detect unfolded and misfolded regions in client proteins. If the protein is incompletely folded or misfolded (poor quality), the folding sensors re-attach it to the folding machinery for re-folding. If the protein is correctly folded (good quality), the folding sensors can no longer detect any disrupted regions in it, which can then exit ER. For glycoproteins (Wang et al. 2015), soon after the addition of the oligosaccharide Glc₃Man₉GlcNAc₂ to the nascent polypeptide chain as it enters ER, the terminal two glucose residues are trimmed by the sequential action of glucosidase I and glucosidase II. The generated monoglucosylated glycan (GlcMan₉GlcNAc₂) can bind to calnexin/calreticulin which, in conjunction with ERp57, helps the correct folding of the glycoprotein. After the removal of the third glucose residue by glucosidase II, the glycoprotein is released from the calnexin/calreticulin cycle and get inspected by the folding sensor UDP-glucose:glycoprotein glucosyltransferase (UGGT). If the protein is improperly folded, UGGT re-attaches one glucose residue to the N-linked glycan, which allows the protein to re-enter the calnexin/calreticulin cycle for further rounds of folding. When the glycoprotein attains its native conformation, UGGT can no longer detect any disrupted regions in it, and thus, the protein can exit ER. For non-glycosylated proteins, folding is assisted mainly by the chaperone BiP (and its co-chaperones). BiP helps the folding of its client substrates via ATP-dependent binding and release cycles (Behnke et al. 2015). In its ATP-bound state, BiP has an open conformation allowing it to bind to substrates. Upon ATP hydrolysis, BiP transforms into a closed conformation which tightly binds to the substrate. Folding occurs when the substrate is released from BiP after the replacement of the bound ADP by a new ATP. If the polypeptide is still incompletely folded or misfolded, BiP recognizes and binds to exposed hydrophobic regions and keeps the protein inside the ER for further rounds of folding. When the protein attains its native conformation, BiP can no longer detect any disrupted regions in it, and thus, the protein can transit to the Golgi. Failure of correct folding renders client proteins as substrates for the ER-associated

degradation (ERAD) machinery (Stevenson et al. 2016), which also binds to misfolded proteins and holds them inside the ER until retrotranslocation into the cytosol for degradation, and prevents their passage to the Golgi.

Importantly, when ER client protein load increases during stress, the activated UPR rapidly upregulates components of the quality control system (Dombroski et al. 2010; Teske et al. 2011; Yamamoto et al. 2007) to bind to the increased amount of immature proteins and to hold them inside ER in a non-aggregated form. Thus, during ER stress, the accumulated immature proteins are not allowed to overwhelm or overflow the quality control barrier (not in yeast—see below). This is supported by a study showing that ER quality control systems remained highly efficient during stress in Chinese hamster ovary (CHO) cells, still allowing only correctly folded proteins to exit ER while holding unfolded proteins inside, without inhibiting the export process (Nadanaka et al. 2004).

With some exceptions, only correctly folded proteins can pass the quality control checkpoint and arrive at exit sites (Mezzacasa and Helenius 2002) where they interact via the correctly presented export determinants with COPII coat, either directly or indirectly, to leave ER. Misfolded proteins in general cannot pass this checkpoint and are kept away from exit sites (Mezzacasa and Helenius 2002) and therefore cannot exit ER even if ER export is highly active. Interestingly, when correctly folded proteins present at exit sites underwent unfolding, they were rapidly recognized by the folding sensors, moved away from the exit sites (back into the reticular ER), and rebound to ER chaperones, further suggesting that immature proteins are not tolerated at exit sites (Mezzacasa and Helenius 2002). Moreover, it was found that only correctly folded proteins can stimulate COPII-vesicle budding from ER membrane, whereas misfolded proteins cannot (Aridor et al. 1999). This indicates that even if some misfolded proteins manage to arrive at exit sites and interact with COPII coat in pre-budding complexes, this will be a non-functional interaction that does not stimulate vesicle budding (Aridor et al. 1999). Thus, misfolded proteins cannot easily exit ER even if ER export is highly active.

The abovementioned exceptions (misfolded proteins capable of exiting ER) are thought to be more common in yeast cells than mammalian cells (Arvan et al. 2002). Yeast cells have a simple and less-regulated ER quality control system, while mammalian cells have a highly developed ER quality control system. Thus, the possibility that the accumulated immature protein will overflow the quality control barrier and escape from the stressed ER is much higher in yeast cells than mammalian cells.

Even if some exportable misfolded proteins manage to pass the quality control barrier and exit ER (by presenting export determinants at ERES such as functional cytoplasmic ER exit signals or being able to bind to export receptors similar to their mature counterparts), other quality control checkpoints along

the secretory pathway such as Golgi quality control can capture them and direct them either back to the ER or toward vacuoles/lysosomes for robust degradation (Arvan et al. 2002; Ashok and Hegde 2009; Kincaid and Cooper 2007; Wang and Ng 2010). Thus, these escaping misfolded proteins are not allowed to fill up post-ER compartments and hinder the functionality of the secretory pathway.

Notably, some misfolded proteins must exit ER as obligate substrates of post-ER quality control such as misfolded Wsc1p (cell wall signaling protein) in yeast (Wang and Ng 2010) and some misfolded mutants of the prion protein in mammals (Ashok and Hegde 2009). These proteins cannot be recognized by the ER quality control systems and cannot be degraded via ERAD and therefore must exit ER for subsequent degradation in vacuoles/lysosomes.

Collectively, these data indicate that it is highly unlikely that the accumulated misfolded proteins will rapidly leave the ER and fill post-ER compartments if ER export remains highly functional during stress. Instead, misfolded proteins will remain localized inside the ER with the help of a strengthened quality control barrier, and the exportable part that could leak from the ER (most commonly in yeast) will be rapidly captured and eliminated by post-ER quality control to keep the post-ER secretory pathway clear. In other words, the cell does not need to inhibit ER export to keep misfolded proteins inside the stressed ER as suggested by Amodio et al.

Argument against inhibition of ER export by ER stress

In fact, there are several observations that argue against inhibition of ER export by ER stress. For example, one study in yeast (Spear and Ng 2003) showed that while overexpression of misfolded carboxypeptidase Y (CPY*) induced ER stress and activated the UPR, it did not inhibit ER-to-Golgi transport of several cargo proteins. Moreover, stress tolerance of overexpressed CPY* required ER exit of some CPY* for subsequent degradation in the vacuole. Inhibiting CPY* exit from the stressed ER was detrimental to the yeast cells. Interestingly, overexpressing CPY* in Δ ire1 cells (which cannot activate the UPR) led to severe inhibition of anterograde transport, indicating that the activation of the UPR could support and maintain efficient ER-to-Golgi protein transport during stress.

Other studies in yeast strongly suggested that the activation of the UPR can positively regulate ER export by establishing that the UPR could rescue the growth of several temperature-sensitive Sec mutants defective in ER export at the restrictive temperature (Higashio and Kohno 2002; Sato et al. 2002).

In mammals, several studies reported that the induction of ER stress had no inhibitory effect on ER export and secretion of some proteins. For example, while tunicamycin treatment inhibited the secretion of thyroxine-binding globulin (which formed intracellular aggregates) from human hepatoma Hep G2 cells, it did not inhibit the secretion of albumin (Bartelena

and Robbins 1984). Similarly, in CHO cells expressing α 1-antitrypsin (A1AT), DTT treatment did not significantly alter its secretion (Nadanaka et al. 2004). In addition, intracellular A1AT did not accumulate inside the stressed ER as would be expected if ER export was inhibited, but instead was found inside Golgi-like structures. In rat hepatoma McA-RH7777 cells, ER stress induced by tunicamycin at doses of 0.2 and 1.0 μ g/ml significantly inhibited the secretion of apoB100 but did not inhibit the secretion of apoB48, apoA-I, or albumin (Ota et al. 2008). At 5.0 μ g/ml dose, tunicamycin reduced the secretion of all these proteins; however, this was consistent with a decrease in total protein synthesis. If ER stress inhibits ER export, total protein secretion via the constitutive secretory pathway should have been inhibited or delayed in all these studies.

Interestingly, other studies reported that triggering ER stress could actually increase the secretion of some other proteins. For example, in one study, treatment of the Raw 264.7 murine macrophages with lipopolysaccharide led to their activation and secretion of interleukin-6 (IL-6) (Kim et al. 2012). Further treatment of these activated cells with tunicamycin significantly increased IL-6 secretion while treatment with brefeldin A (which inhibits ER-to-Golgi protein trafficking) inhibited its secretion. If ER stress inhibits ER export, then tunicamycin should have decreased, rather than increased, IL-6 secretion similar to the effect of BFA. Increased secretion by tunicamycin is more consistent with the enhancement of ER export (and probably the entire secretory pathway) by ER stress. Similarly, ER stress induced by tunicamycin or thapsigargin markedly enhanced the secretion of IL-23 from monocyte-derived dendritic cells stimulated with Toll-like receptor agonists (Goodall et al. 2010). Both IL-6 and IL-23 are secreted via the constitutive secretory pathway. ER stress was shown also to increase the secretion of pentraxin 3 from ARPE-19 human retinal pigment epithelial cells (Hwang et al. 2016) and the secretion of prostate apoptosis response-4 (Par-4) protein from PC-3 prostate cancer cells (Burikhanov et al. 2009). Finally, the secretion of vascular endothelial growth factor (VEGF) from several human tumor cell lines was shown to increase during ER stress induced by glucose deprivation, thapsigargin, or tunicamycin (Wang et al. 2012).

In addition to correctly folded proteins, ER stress was shown to rapidly increase the export of a subset of misfolded proteins (GPI-anchored proteins) from the ER for subsequent degradation in lysosomes via the proposed RESET (rapid ER stress-induced export) pathway (Satpute-Krishnan et al. 2014), also arguing against inhibition of ER export during stress to keep misfolded proteins inside the ER. This also suggests that increased export of specific misfolded proteins from the ER during stress is an important mechanism to decrease ER misfolded protein load, indicating that active ER export could participate in relieving stress.

It is known that during the activation of the UPR, ATF6 is transported from the ER to the Golgi where it is cleaved and activated (Grootjans et al. 2016). One study (Teske et al. 2011) revealed that efficient activation of the ATF6 pathway is dependent on the activation of the PERK pathway. The authors found that the activation of the PERK/phospho-eIF2 α /ATF4 pathway during stress contributed to increased expression of genes involved in ER-to-Golgi transport, and this was suggested to facilitate ATF6 transport from the ER to the Golgi. Knockout of PERK (which inhibited this increase in gene expression) led to a dramatic decrease in ATF6 activation. This suggests that the PERK-dependent upregulation of ER-to-Golgi transport machinery and the resulting enhancement of anterograde protein transport during stress are prerequisites for efficient activation of the ATF6 branch of the UPR, also arguing against inhibition of ER export by ER stress. In the same study (Teske et al. 2011), when the ER of wild-type MEF cells was stressed by 6-h exposure to tunicamycin or thapsigargin, ATF6 was transported to the Golgi and the cleaved form ATF6(N) appeared early after 1-h exposure, indicating rapid activation of the UPR. During the remaining treatment regimen, ATF6 molecules kept traveling from the ER to the Golgi for activation, indicating that ER export remained active during the entire stress induction period (6 h).

If ER stress inhibits ER export, then an important question arises: why does the UPR upregulate components of the export machinery? The cell should better downregulate the export machinery to help inhibit ER export. In general, upregulation of a specific component of the secretory pathway during stress enhances its functionality. For example, the upregulation of the ERAD machinery by the UPR enhances ERAD activity (Doroudgar et al. 2015). Similarly, the upregulation of the ER export machinery by the UPR could be speculated to increase the functionality of this part of the secretory pathway. However, this needs to be confirmed experimentally.

Another important question also arises: what happens to proteins that become correctly folded by the upregulated folding machinery and arrive at exit sites during stress? If ER stress inhibits the export process, then these proteins should accumulate inside ER. Can the accumulation of these correctly folded proteins affect ER homeostasis? In fact, studies showed that inhibiting protein transport from the ER to the Golgi in non-stressed cells and the resulting accumulation of correctly folded proteins inside the ER can itself trigger ER stress (Fang et al. 2015; Hikiji et al. 2015; Preston et al. 2009). Hence, inhibition of ER export in stressed cells should exacerbate or at least maintain, rather than alleviate, ER stress. This suggests that for the enhanced folding machinery to alleviate ER stress, a parallel enhancement of ER export is needed to rapidly export proteins arriving from the folding chamber to exit sites.

During chondrogenesis, differentiating chondrocytes secrete large amounts of extracellular matrix proteins (ECM) and thus encounter ER stress. One study showed that the ER stress-

induced increase in Sec23a expression (through the BBF2H7–Sec23a pathway) was required to support the high secretory function of these cells (Saito et al. 2009), supporting the idea that ER export is enhanced during stress. The absence of this increase in Sec23a expression (in Bbf2h7^{−/−} chondrocytes) was shown to result in decreased secretion of ECM proteins, their accumulation inside ER, exacerbation of ER stress (as mentioned above), and abnormal cartilage formation, reflecting the importance of maintaining and improving ER export function during stress to support normal cartilage formation and to tolerate the existing stress inside chondrocytes.

All the points mentioned above support the notion that ER stress itself does not inhibit ER export. Instead, the UPR may enhance ER export and ER-to-Golgi transport. This is directly supported by the study of Farhan et al. (2008) under comparison which showed that the activation of the UPR was associated with an increase in ERES number during stress. Notably, in the same study, the induction of ER stress without the activation of the UPR (in IRE1^{−/−} MEFs) did not decrease the number of ERES, indicating no direct inhibitory effect of ER stress on ER export.

These points also reveal that the maintenance and enhancement of ER export activity during stress may serve several purposes including (a) maximal activation of the ATF6 branch of the UPR, (b) rapid and efficient evacuation of cargo proteins from the ER after correct folding to relieve stress and to support high secretion, (c) rapid reduction of misfolded protein load inside the ER by allowing rapid export of some misfolded proteins (such as GPI-anchored proteins) to the Golgi for subsequent degradation in lysosomes, and (d) in yeast, functional ER export during stress can also allow part of the exportable misfolded proteins to travel to the Golgi before degradation, enabling cells to tolerate stress.

Why did ER export appear inhibited during pharmacologically induced ER stress?

One important difference between the two sets of experiments under comparison is the method of ER stress induction. In the studies of Amodio et al. (2009, 2013), ER stress was induced using the pharmacological agents TG, DTT, or MG123. In study of Farhan et al. (2008), prolonged overexpression of a cargo protein induced ER stress.

TG is a sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) inhibitor which depletes ER Ca²⁺. Normally, the maintenance of high Ca²⁺ content inside the ER is essential for proper functionality of chaperones and folding enzymes during protein folding (Corbett and Michalak 2000; Gidalevitz et al. 2013). ER Ca²⁺ depletion can disrupt the functionality of the folding machinery and inhibit correct protein folding (Pétremand et al. 2012; Preston et al. 2009), leading to the accumulation of misfolded proteins inside the ER and reducing the amount of correctly folded proteins available for export.

The ER lumen has a redox environment that is different from other cellular compartments (more oxidizing) and is highly regulated to support disulfide bond formation in newly translocated proteins with the aid of folding enzymes (Hatahet and Ruddock 2009; Margittai and Sitia 2011). Exposure of cells to the strong reducing agent DTT can disrupt this environment, inhibit new disulfide bond formation, and reduce already formed bonds in folding intermediates, leading to the accumulation of immature proteins inside the ER and reduces the amount of correctly folded proteins available for export (Braakman et al. 1992; Negroiu et al. 2000; Tatu et al. 1993).

MG132 is a proteasome inhibitor that causes the accumulation of several ERAD substrates inside ER. Some studies showed that inhibition of proteasomal function could rapidly activate a specific eIF2 α kinase (other than PERK) which rapidly phosphorylated eIF2 α resulting in a significant decrease in protein synthesis even with minimal induction of ER stress (Jiang and wek 2005; Yerlikaya et al. 2008). This early decrease in protein synthesis can reduce the amount of mature proteins available for ER export during subsequent ER stress. Additionally, studies also showed that, upon proteasomal inhibition, several ERAD substrates were retained inside the ER in prolonged association with chaperones such as BiP and calnexin (Chillarón and Haas 2000; Elkabetz et al. 2004; Mancini et al. 2000; Oda et al. 2003). As time passes, the buildup of these ERAD substrates inside the ER can sequester more chaperones, which thus cannot assist the folding of newly translocated proteins. This, in turn, can decrease the capacity of the folding machinery to generate transport-competent mature proteins.

Collectively, it appears that all the agents used to induce ER stress in the studies of Amodio et al. can disrupt the folding machinery/environment and decrease the generation of transport-competent mature proteins inside ER. In contrast, in the study of Farhan et al. (2008), ER stress was induced via overloading the folding machinery, which was still highly functional and further enhanced by the UPR and was capable of generating increased amounts of transport-competent mature proteins.

In fact, it was found that the extent of ER export depends mainly on the amount of transport-competent proteins inside the ER and that changes in this amount can directly alter the extent of ER export. For example, in one study (Aridor et al. 1999), modulating the amount of exportable cargo inside the ER (using the temperature-sensitive variant of vesicular stomatitis virus G protein in tsO45 VSV-infected cells or using the PiZ variant of A1AT vs. wild-type A1AT) was shown to alter the number of buds formed on the ER membrane. Decreasing the amount of exportable cargo led to a decrease in the number of buds and vice versa. In the same study, inhibiting protein synthesis using cycloheximide (which reduces the amount of exportable cargo) also led to a decrease in the number of ER-derived buds. This can explain the effect of MG132 (which also rapidly inhibits protein synthesis)

which decreased COPII binding to the ER membrane in the second study of Amodio et al. (2013). The dependence of ER export on the amount of transport-competent cargo is also evident in yeast (Kaiser and Schekman 1990) and plant cells (Hanton et al. 2007).

Interestingly, reducing the amount of exportable proteins inside the ER was shown also to accelerate Sec23p turnover at ERES (Forster et al. 2006). This is similar to the effect of thapsigargin which also accelerated Sec23a turnover at ERES in the second study of Amodio et al. (2013), and suggests that this reduced stability of Sec23 protein on ER membrane resulted not from TG-induced ER stress itself but rather from decreased generation of exportable proteins after TG-induced disruption of protein folding.

Collectively, these data suggest that ER export appeared inhibited during pharmacologically induced ER stress in the studies of Amodio et al. due to the inhibitory effect of stress-inducing agents on the generation of transport-competent proteins inside ER. In general, the activation of the UPR may improve the ER export environment during stress (via certain mechanisms). If ER stress is induced via overloading the intact folding machinery (with correctly folded proteins), the generation of transport-competent proteins will increase and thus ER export will appear increased (Fig. 1a). If ER stress is induced via disrupting the folding machinery/environment, the generation of transport-competent proteins will decrease and thus ER export will appear reduced (Fig. 1b).

Importantly, other agent-related effects (other than ER stress) may also influence anterograde transport during stress. For example, ER luminal Ca^{2+} depletion per se was shown to inhibit ER-to-Golgi transport of correctly folded and

oligomerized proteins without activation of the UPR (Helm et al. 2014), reflecting the dependence of the transport process on luminal Ca^{2+} . This is particularly relevant to the first study of Amodio et al. (2009) where TG treatment (which induces both ER Ca^{2+} depletion and ER stress) inhibited anterograde transport of transport-competent proteins and suggests that this inhibition did not result from ER stress itself. Notably, not all the agents used in the studies of Amodio et al. have such off-target effects on anterograde protein transport. A couple of studies showed that treatment of cells with DTT did not inhibit the secretion of the disulfide bond-free A1AT, indicating that DTT has no direct inhibitory effect on ER export or the distal part of the secretory pathway (Lodish and Kong 1993; Nakanaka et al. 2004). This is why DTT is recommended to use during confirmation (see “Guidelines for confirmation” section) as it only disrupts protein folding.

A proposed mechanism for ER export enhancement by the UPR

Several studies reported that the induction of ER stress can rapidly activate (phosphorylate) the MAP kinases ERK1/2 (Arai et al. 2004; Bobrovnikova-Marjon et al. 2012; Hu et al. 2004; Zhang et al. 2009). One study examining the effects of various kinases and phosphatases on the early secretory pathway identified a positive relationship between the activity of the MAP kinase ERK2 and the number of ERES and also ER-to-Golgi protein transport (Farhan et al. 2010). Increasing the activity of ERK2 led to an increase in ERES number and vice versa. Similarly, increased activity of ERK2

Fig. 1 The integrity of the folding machinery/environment determines whether ER export appears increased or decreased during stress. **a** When the folding machinery/environment is intact (green color), the generation of correctly folded proteins (transport competent) will increase, which in turn stimulate the formation of more export vesicles,

and thus, ER export will appear enhanced. **b** When the folding machinery/environment is impaired (red color), the generation of correctly folded proteins will decrease, leading to the formation of fewer export vesicles, and thus, ER export will appear inhibited. The UPR enhances the ER export environment in both cases

in ER-stressed cells is expected to increase ERES number and enhance ER export.

The ERES protein Sec16 was shown to be an ERK2 target, and ERK2-induced phosphorylation of Sec16 was suggested to be responsible for the increase in ERES number (Farhan et al. 2010). This may provide a mechanistic basis for ER export enhancement by the UPR via two pathways: transcriptional upregulation of the export machinery (transcriptional pathway) and ERK2-induced phosphorylation of Sec16 which increases ERES number (phosphorylation pathway) (Fig. 2).

One study (Bobrovnikova-Marjon et al. 2012) proposed a pathway for ERK1/2 activation during ER stress in which phosphatidic acid produced due to the lipid kinase activity of PERK can trigger the Ras-MEK-Erk1/2 signaling pathway. Another study (Zhang et al. 2009) suggested that ERK1/2 activation is partially dependent on IRE1 α signaling pathway of the UPR (Fig. 2). The latter is consistent with the lack of increase in ERES number during ER stress in IRE1 $^{-/-}$ MEFs in the study of Farhan et al. (2008) under comparison.

If PERK is responsible for ERK activation, then PERK could be regulating both protein entry to the ER and protein exit from the ER during stress via the protein kinase activity (which phosphorylates eIF2 α) and the lipid kinase activity (which activates ERK2), respectively (Fig. 2).

For the transcriptional pathway, multiple arms of the UPR appear to contribute to the upregulation of genes involved in ER export and ER-to-Golgi transport. For example, one study suggested that the PERK arm may contribute to such upregulation, as knockout of PERK was shown to attenuate the upregulation of a number of these genes during stress (Teske et al. 2011). In another study, XBP1 was shown to bind to several of these genes under various levels of UPR activation (Acosta-Alvear et al. 2007), suggesting that the IRE-1 arm could also be involved. In chondrocytes, the UPR transducer BBF2H7 was shown to bind to Sec23a promoter and increase

its transcription during ER stress (Saito et al. 2009). Importantly, attention should be given to the cross-regulation between the UPR arms, as the loss of activity of one arm (such as PERK) can attenuate the magnitude of activity of other arms (ATF6 and XBP1s) during stress (Fusakio et al. 2016; Majumder et al. 2012; Teske et al. 2011).

Notably, since all the three arms of the UPR could remain activated to some level in cells adapted to chronic ER stress (Rutkowski et al. 2006), anterograde transport machinery may remain upregulated and ER export may remain enhanced by the chronically activated UPR. After all, prolonged enhancement of ER export is compatible with cellular function (unlike the UPR-induced translational inhibition, which must be reversed). Additionally, long-term improvement of ER protein processing capacity during chronic stress (Rutkowski et al. 2006) necessitates a parallel long-term improvement of ER export activity to efficiently export any amounts of proteins generated by the intact folding machinery. This would maintain the new balance established inside the ER by the activated UPR to enable cellular adaptation and survival. Inhibiting ER export during chronic stress should disrupt this new balance, exacerbate the stress response, and compromise cellular adaptation. This may be helpful for the treatment of diseases in which adaptation to chronic ER stress occurs such as some types of cancer (Corazzari et al. 2015).

The need to improve the ER export activity in parallel with the improvement of ER protein processing capacity to preserve ER balance could be a general phenomenon that applies to different cell types. For example, professional secretory cells such as plasma cells are thought to have a constitutively active UPR (Acosta-Alvear et al. 2007) to maintain a highly upregulated protein processing machinery inside the ER to support the synthesis and secretion of large amounts of proteins. In these cells, XBP1 was shown to bind to several genes involved in ER export and anterograde transport (Acosta-Alvear et al. 2007), suggesting that the constitutively active

Fig. 2 The UPR could regulate protein entry to ER and protein exit from ER during stress. Protein entry: the activation of PERK (protein kinase activity) phosphorylates eIF2 α leading to the inhibition of new protein synthesis. Protein exit: the activation of IRE1 or PERK (lipid kinase activity) leads to the phosphorylation of ERK2 which in turn phosphorylates Sec16 resulting in the formation of more ER exit sites (phosphorylation pathway). The UPR also upregulates the export machinery (transcriptional pathway)

UPR also maintains a highly improved ER export function to enable high protein synthesis without the induction of severe ER stress.

Guidelines for confirmation

Brefeldin A is an inhibitor of ER-to-Golgi protein transport and causes the accumulation of newly synthesized proteins inside ER. However, it does not inhibit the assembly of COPII coat at ERES (Ward et al. 2001). Prolonged exposure of cells to BFA (4–12 h) can trigger ER stress (Crespo et al. 2012; Rab et al. 2007; Zhou et al. 2013).

The proposed assumption can be tested by measuring the change in ER export in cells stressed with BFA alone (ER stress without disruption of the folding machinery/environment) and cells stressed with BFA and DTT (ER stress with disruption of the folding machinery/environment). In the case of BFA alone, new transport-competent proteins will continue to form and accumulate inside ER during the treatment regimen, while in the case of BFA and DTT, no new transport-competent proteins will be formed inside ER. Thus, at the end of the treatment regimen, the amount of transport-competent proteins should be larger in the first case than the second case. According to the presented assumption, ER export should appear increased in the first case compared to the second case. If ER stress inhibits the export process, then ER export should appear decreased in both cases regardless of the amount of exportable proteins present inside ER.

Alternatively, a non-pharmacological approach includes measuring the change in ER export in cells stressed by overexpression of a correctly folded protein and cells stressed by overexpression of the misfolded mutant form of the same protein. The generation of transport-competent proteins inside ER should be higher in the first case than the second case. Accordingly, ER export should appear increased in the first case compared to the second case. If ER stress inhibits the export process, then ER export should appear decreased in both cases.

Conclusions

It appears that the UPR enhances ER export function as one mechanism to decrease ER protein load and relieve ER stress. However, whether ER export will appear increased or decreased during stress depends on the integrity of the folding machinery/environment inside the stressed ER. Multiple arms of the UPR appear to participate in enhancing ER export during stress. Manipulating this response to alter cell fate during stress may be useful for the treatment of ER stress-related diseases.

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