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The oligomeric state of Derlin-1 is modulated by endoplasmic reticulum stress

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Abstract
The endoplasmic reticulum (ER) is a major site of protein synthesis in eukaryotes. Newly synthesized proteins are monitored by a process of quality control, which removes misfolded or unassembled polypeptides from the ER for degradation by the proteasome. This requires the retrotranslocation of the misfolded proteins from the ER lumen into the cytosol via a pathway that, for some substrates, involves members of the recently discovered Derlin family. The Derlin-1 isoform is present as a dimer in the ER, and we now show that its dimerization is modulated by ER stress. Three distinct types of chemically-induced ER stress substantially reduce the levels of Derlin-1 dimer as assayed by both cross-linking and co-immunoprecipitation. The potential function of the different Derlin-1 populations with respect to ER quality control is investigated by analysing their capacity to associate with a misfolded membrane protein fragment. We show for the first time that Derlin-1 can associate with an aberrant membrane protein fragment in the absence of the viral component US11, and conclude that it is the monomeric form of Derlin-1 that interacts with this potential ER-associated degradation substrate. On the basis of these data we propose a model where the pool of active Derlin-1 in the ER membrane can be modulated in response to ER stress.

Keywords: Quality control, ER stress, ER associated degradation

Introduction
When newly synthesized membrane and secretory proteins fold incorrectly they are recognized by the ER quality control machinery and targeted for degradation. This process typically involves ubiquitination, retrotranslocation across the ER membrane and deglycosylation prior to degradation at the proteasome [1,2]. Several studies suggest that retrotranslocation might occur via the Sec61 import machinery operating in reverse [3,4]. However, other ER components contribute to the extraction of MHC class I heavy chain from the bilayer in a process facilitated by specific viral proteins [5,6]. Amongst these is Derlin-1, an ER membrane protein with four transmembrane domains that might act as an alternative site for retrotranslocation across the ER membrane [7]. The possibility that Derlin-1 might form a retrotranslocation channel is consistent with the observation that it can form a dimer, although it is also found in a complex with several other components implicated in quality control at the ER [5,6]. These include subunits of a ubiquitin ligase complex (HRD1 and SEL1), the p97 ATPase (also known as VCP) and VCP-interacting membrane protein (VIMP). All of these proteins have been shown to co-immunoprecipitate as part of larger complexes that can be recovered from digitonin solubilized cell lysates [8,9], and these complexes may act as a channel for the selective removal of unwanted proteins from the ER. More recently the Derlin-1-related proteins Derlin-2 and Derlin-3 have been implicated in the ER-associated degradation (ERAD) pathway that removes misfolded glycoproteins from the ER in the absence of any co-expressed viral co-factors [10].

As nascent polypeptides emerge into the ER, their maturation, folding and assembly is normally assisted by numerous luminal chaperones [11]. However, should levels of misfolded proteins exceed the “folding capacity” of the ER, a state of stress that triggers the unfolded protein response (UPR) occurs [12]. The UPR provides an ER-to-nucleus signalling cascade that can upregulate chaperone production to enhance the clearance of misfolded proteins from the...
ER and consequently alleviate ER stress. In mammalian cells, the UPR is driven by at least three sensors: PKR-like endoplasmic reticulum eIF2α kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1) [13,14]. Activated PERK phosphorylates eIF2α, reducing the rate of protein synthesis and thereby the load on the secretory system. ATF6 activation alters the transcription of numerous target genes, including XBP-1, whilst activated IRE1 splices the resulting XBP-1 mRNA causing the expression of a protein with an additional transactivation domain [15,16]. The resulting spliced XBP-1 product is a potent transcription factor that upregulates the expression of ER chaperones and promotes the biogenesis of ER membrane [15,17–19]. A clear link between the expression of the Derlins and ER stress has been established [6,10], and in this study we set out to examine whether, in addition to its gene expression, the molecular environment of Derlin-1 could respond to ER stress. Using a cross-linking approach, we show that the proportion of Derlin-1 present as a dimer is significantly reduced following ER stress. We recapitulate this result using a co-immunoprecipitation strategy, thereby confirming that ER stress results in a true loss of dimer and not a conformational change that influences cross-linking efficiency.

We conclude that a fraction of Derlin-1 is normally present in the ER as a homodimer, and that this homodimeric Derlin-1 is disrupted as a consequence of ER stress. This would seem at odds with the possibility that homodimers of Derlin-1 contribute to a retrotranslocation channel. In order to address the potential functions of the different oligomeric forms of Derlin-1, we therefore investigated their association with a model misfolded membrane protein fragment [20]. We provide the first evidence for a US11-independent interaction between Derlin-1 and a potential ERAD substrate, and find that the monomeric form of Derlin-1 is associated with this polypeptide. On this basis, we propose a model where ER stress releases Derlin-1 from an inactive homodimeric complex, providing a potential mechanism for the cell to modulate its ERAD capacity.

Materials and methods

Antibodies and reagents

Anti-V5-HRP and anti-myc agarose affinity gel were from Sigma, and anti-HA affinity matrix from Roche. The mouse monoclonal antibody against α-Tubulin (TAT-1) was a gift from Keith Gull (University of Oxford, UK). Derlin-1 and SPP specific polyclonal rabbit antisera were custom made by Eurogentec (Seraing, Belgium) and the goat anti-rabbit IgG-HRP used for immunoblotting was from Sigma (Poole, UK). The monoclonal antibody specific for the N-terminus of bovine opsin was a gift from Paul Hargrave (University of Florida, USA). The cross-linking reagent, bismaleimidohexane (BMH) was from Pierce (Chester, UK). Easytag L-[^35]S)methionine/cysteine was purchased from NEN Dupont (Stevenage, UK). Reagents for cell culture were obtained from Invitrogen (Paisley, UK), whilst all other chemicals were purchased from BDH/Merck (Poole, UK) and Sigma.

Opsin derived constructs

Bovine opsin derivatives containing a single cysteine at residue 56 (OP[cys56]) are as previously described [20,21]. Templates for the transcription of truncated opsin mRNAs were prepared by PCR [22]. Forward primers were located 160 bases 5’ of the RNA polymerase promoter, whilst reverse primers were designed to generate truncations encoding the N-terminal 91 amino acids of opsin. No stop codon was present in the resulting mRNAs and the majority of the resulting polypeptides remain attached to the ribosome unless released by treatment with puromycin [21–23]. PCR products were purified directly from the reaction mixture using the QIAquick PCR purification kit (Qiagen, Crawley, UK).

Transcription, translation and cross-linking

Transcriptions were carried out using T7 RNA polymerase as described by the manufacturer (Promega) and RNA purified using the RNeasy RNA purification kit (Qiagen) before use in translation reactions. Cultured HeLa cells or HT-1080 fibroblasts (ATCC CCL-2 and CCL-121 respectively, American Type Culture Collection, Rockville, MD, USA) were semi-permeabilised with the detergent digitonin (Calbiochem, Nottingham, UK) as previously described [24]. RNA encoding opsin fragments [20] was translated in a rabbit reticulocyte lysate system (Promega) supplemented with L[^35]Smethionine/cysteine and semi-permeabilized HT1080 cells as a source of ER membrane. After 15 min at 30°C aurintricarboxylic acid was added (100 μM final concentration) to inhibit translation initiation and after another 10 min samples were treated with 2 mM puromycin and 50 mM EDTA.
for 10 min at 30°C to release the ribosome from the nascent chain.

Following translation, membrane associated polypeptides were recovered from the translation mix by centrifugation for 10 sec at 16,000 g and the resulting membrane fraction washed twice by resuspension in KHM buffer (110 mM KOAc, 2 mM MgOAc, 20 mM HEPES pH 7.2). The membrane pellet was then resuspended in KHM and the cross-linking reagent BMH added to a final concentration of 1 mM. BMH cross-links adjacent proteins via the –SH groups of available cysteines. Samples were incubated at 30°C for 10 min and the cross-linking reaction quenched by the addition of 0.1 volumes of 100 mM 2-mercaptoethanol and incubation on ice for 10 min. All samples were treated with RNase A (7 μg/translation reaction) to remove any tRNA that remained attached to stalled polypeptide chains prior to SDS-PAGE [23]. As previously observed [21,22], the truncated opsin fragment was correctly membrane inserted, and efficient glycosylation of the asparagine residues at positions 2 and 15 of the N-terminus of the OP91 polypeptide was detected.

**Transient expression, cross-linking and immunoblotting**

Derlin-1 was amplified from an IMAGE cDNA clone (IMAGE ID: 3345230) and subcloned into the mammalian expression vector pcDNA5/FRT/ V5-His using the TOPO TA expression system to produce a derivative with a V5-epitope tag at the C-terminus. The V5 tag was subsequently altered by PCR to generate an HA tagged version of Derlin-1 in the same vector. These constructs were transiently transfected into HeLa or HT-1080 cells using Lipofectamine 2000 (Invitrogen) as specified by the manufacturer. After overnight incubation, wells containing ~2 x 10^7 cells were rinsed twice with PBS and then 3 ml of 0.25 mM BMH (freshly dissolved in PBS) was added per 10 cm-dish of HeLa cells. After 10-min incubation at room temperature, the 3 ml solution of cross-linker was aspirated and 400 μl sample buffer was added to quench the reagent and harvest the cells. This viscous mixture was homogenized by sonicating for 15 min in a Diagenode Bioruptor UCD-200 waterbath (Liège, Belgium). Where material was immunoprecipitated from the transfected HeLa cells, the cross-linking reagent was quenched by the addition of 100 μl 250 μM cysteine and methionine, followed by washing twice in PBS. 500 μl of Triton X-100 immunoprecipitation buffer (10 mM Tris/HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% Triton X-100) was then added and the cells left to solubilise for 15 min at room temperature, followed by immunoprecipitation with either the anti-HA affinity matrix or a control antibody. The immunoprecipitated material, together with a sample of the input lysates (one-tenth) was resolved by SDS-PAGE, and the resulting products analysed by Western blotting.

**Immunoprecipitation**

Denaturing immunoprecipitations were performed by heating the quenched, cross-linked, samples for 30 min at 37°C in the presence of 1% SDS. Four volumes of Triton X-100 IP buffer were then added and the samples were incubated on ice for ~30 min, followed by centrifugation at 16,000 g for 5 min. Aliquots of the resulting supernatant were gently agitated overnight at 4°C with the relevant antisera in the presence of 200 μg/ml phenylmethylsulfonyl fluoride and 1 mM cysteine and methionine. Protein A-Sepharose was added and the incubation continued for 2 h, after which Protein A-Sepharose-bound material was isolated by centrifugation at 16,000 g for 1 min, washed four times with Triton X-100 IP buffer and then heated to 37°C for 30 min in SDS-PAGE sample buffer.

**Sample analysis**

Radioactive cross-linking products from in vitro translations were analysed on 14% SDS-polyacrylamide gels, before drying the gels and exposing for three days to a phosphorimaging plate for visualization on a Fuji BAS 3000 phosphorimaging system. For immunoblotting, gels were transferred to Hybond C membrane (Amersham Pharmacia, Chalfont St. Giles, UK) and visualized using an enhanced chemiluminescence system (Western Lightning, PerkinElmer, Boston, USA). Quantitative analysis of gels was carried out using AIDA version 3.44 (Raytest Isotopenmessgerate GmbH, Straubhardt, Germany). Briefly, product intensities were measured and used to calculate the proportion of Derlin-1 cross-linked in a dimeric form after different drug and chemical treatments. This was done by determining the amount of Derlin-1 cross-linked dimer in BMH-treated samples, and expressing it as a fraction of the Derlin-1 monomer seen in the absence of cross-linker. These data were then compared to control experiments to indicate relative efficiencies of dimer formation after different treatments. The mean and the standard error of the mean were calculated and Figure 2B drawn using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, San Diego, USA).
Results

ER stress reduces Derlin-1 dimerization

Two recent studies have suggested that Derlin-1 is normally present in the ER membrane as a homodimer [8,9] and using both co-immunoprecipitation strategies and bifunctional cross-linking approaches we also find evidence that a significant amount of Derlin-1 is present in the ER membrane as a homodimer (data not shown). Having tested several bifunctional cross-linking reagents to examine the dimerization status of Derlin-1 we concluded that BMH was the most useful for subsequent analysis (see Figure 1).

Whilst dimeric forms of Derlin-1 have been previously described [8,9], the functional significance of these homodimers is unclear. In order to better understand their role in relation to ER quality control, we investigated how the oligomeric status of Derlin-1 responded to ER stress. In order to induce a significant level of ER stress we initially exposed HeLa cells to the reducing agent DTT, which is known to generate a robust stress response via the actions of ATF6 [25–28]. When the oligomeric status of Derlin-1 was analysed following DTT treatment, the amount of the 46 kDa BMH dependent homodimer obtained was reduced to a mean of 43% of that seen in the control cells (Figure 1A, cf. lanes 2 and 4, dimer; see Figure 2B for quantification).

Figure 1. Derlin-1 oligomeric status is altered during DTT-induced ER stress. (A) HeLa cells were grown to ~90% confluency and an UPR was induced by addition of 2 mM DTT every hour for 5 h. DTT was washed out with PBS prior to treatment with the cross-linking reagent BMH at a final concentration of 250 μM. Samples were resolved on a 14% SDS polyacrylamide gel, transferred to nitrocellulose by semi-dry blotting, and incubated with a rabbit anti-Derlin-1 serum. Products were visualized by ECL-based detection. The Derlin-1 monomer was detected at ~28 kDa (lanes 1–4, monomer) whilst BMH dependent dimers were at ~46 kDa (lanes 2 and 4, dimer). (B) RNA isolated from DTT and tunicamycin treated cells was used for cDNA synthesis and RT-PCR. Primers specific for an internal region of XBP-1 generated either a 214 bp product from the spliced XBP-1 mRNA or a 240 bp product from the unspliced mRNA. The RT-PCR products were separated on a 2% agarose gel.

Figure 2. Derlin-1 dimerization is reduced by distinct inducers of ER stress. (A) HeLa cells grown to ~90% confluency were incubated with 2 μM thapsigargin overnight. Cells were then treated with BMH or a solvent control, harvested and analysed using SDS-PAGE and immunoblotting. (B) Bar chart illustrating the mean relative intensity of homodimer seen after BMH treatment in cells treated with the different chemical ER stress-inducers (number of independent experiments performed was 4, 5 and 4 for DTT, tunicamycin and thapsigargin treatments respectively). Error bars represent the standard error of the mean.
tion). The DTT treatment used did not reduce the levels of monomeric Derlin-1 present (Figure 1A, cf. lanes 1–2 and 3–4, monomer) suggesting the effect was due to loss of the dimer rather than a loss of Derlin-1 per se. RT-PCR confirmed that DTT treatment resulted in XBP-1 mRNA splicing indicative of classical UPR induction [15,16] (Figure 1B, lane 3), whilst the untreated cells showed no such splicing (Figure 1B, lane 1). The simplest explanation of this observation is that the levels of Derlin-1 homodimer are reduced in response to the ER stress that results from DTT treatment. If this were true, then one would expect other methods of generating ER stress to have a similar effect upon the oligomeric status of Derlin-1.

In order to establish whether there is a generic link between ER stress and Derlin-1 homodimerization, we used two other well-characterized mechanisms of inducing ER stress, namely treatment with the ER Ca\(^{2+}\) ATPase inhibitor, thapsigargin, or an inhibitor of N-linked glycosylation, tunicamycin. After an overnight treatment with thapsigargin, HeLa cells again showed a clear reduction in the levels of Derlin-1 homodimer detectable after treatment with BMH (Figure 2A, cf. lanes 2 and 4, dimer). In this case the mean reduction resulting from thapsigargin treatment was to 58% of the matched control (Figure 2B). In contrast, the levels of cytosolic α-Tubulin present in the cells were unaltered upon treatment with thapsigargin (Figure 2A, lower panel). Treatment with tunicamycin also resulted in a substantial reduction in the amount of Derlin-1 homodimer recovered upon BMH-mediated cross-linking, in this case the Derlin-1 dimer was present at a mean relative intensity of 49% compared to the level seen in control cells (Figure 2B). In contrast, the levels of the Derlin-1 monomer and the ER-resident chaperone calreticulin were not significantly affected (data not shown). Treatment with tunicamycin did not result in XBP-1 splicing (Figure 1B, lanes 1–2), although the drug was clearly effective since it completely inhibited the ability of the HeLa cells to N-glycosylate a model precursor [20,21] when analysed in a cell free system (data not shown). We therefore conclude that like DTT treatment, the ER stress resulting from thapsigargin and tunicamycin treatments [13] can also cause a marked reduction in the levels of Derlin-1 homodimer present in mammalian cells. Our analysis clearly shows that XBP-1 splicing is not required for the loss of homodimer observed, and we conclude that alternative stress response pathways are most likely also involved [29].

We had now shown that three distinct methods of inducing ER stress all led to a significant reduction in the level of Derlin-1 dimer present in mammalian tissue culture cells. Our interpretation of this result was that the Derlin-1 dimer dissociated as a consequence of ER stress in a manner akin to that suggested for the dissociation of the ER luminal chaperone BiP from the ER stress signalling component IRE1 [30]. However, an alternative explanation of our observations thus far was that the effect of ER stress was to cause an alteration in the conformation of Derlin-1 that reduced the cross-linking efficiency observed but did not affect the protein's dimeric status. In order to address this issue directly, we exploited the fact that the Derlin-1 dimer is stable in a variety of detergents (data not shown, see also [8,9]). We found that treatment with tunicamycin substantially reduced the amount of V5-tagged Derlin-1 co-immunoprecipitated with the HA-tagged form such that only 55% (standard error 4.9% based on three independent experiments) of that recovered in the control cells was obtained (Figure 3, cf. lanes 2 and 5). We therefore conclude

Figure 3. Co-immunoprecipitation confirms that Derlin-1 dimerization is reduced by ER stress. V5- and HA-tagged Derlin-1 were co-transfected into HeLa cells and transiently expressed overnight in the presence of 20 μg/ml tunicamycin or a solvent control. Cells were solubilized in buffer containing 1% Triton X-100, and used for either an HA or a control (myc) immunoprecipitation. Samples were then analysed by SDS-PAGE together with a sample of the input lysates (one-tenth) and immunoblotted with the V5-HRP antibody.
that ER stress results in a loss of Derlin-1 homodimers and that this effect is directly reflected by a reduction in the yield of cross-linking product.

Derlin-1 associates with potential ERAD substrates in vitro

The proposed function of Derlin-1 is to facilitate the ER associated degradation of a subset of misfolded membrane proteins, although the identity of its endogenous substrates and its precise mechanism of action are unclear [5,6,8,9]. To address this issue further, we first investigated whether a fragment of opsin (OP91) that we had previously defined as a potential ERAD substrate [20] displayed any association with Derlin-1 in vitro. We found that when OP91 was synthesized in a cell-free system supplemented with semi-intact mammalian cells, a single cysteine probe within the OP91 polypeptide could be cross-linked to Derlin-1 and the resulting adduct identified by immunoprecipitation with two different antisera specific for Derlin-1 but not a control serum (Figure 4A, cf. lanes 1, 2, 4–6). When the same experiment was carried out using mammalian cells transiently expressing a V5 epitope tagged form of Derlin-1, the epitope tagged form of Derlin-1 was found to out-compete the wild type version and the resulting cross-linking product with the OP91 polypeptide was efficiently recovered with a monoclonal anti-V5 antibody (Figure 4B, lane 5), whilst adducts with the endogenous Derlin-1 were now barely visible (Figure 4B, lane 4).

We also considered the oligomeric form of Derlin-1 that was cross-linked to the OP91 fragment in the in vitro system. As previously, we found that OP91 formed adducts with both monomeric and dimeric forms of the ER component signal peptide peptidase (SPP) and both of these products could be identified by cross-linking (Figure 4A and 4B, lane 3; see also [20]). In contrast, we could only find evidence for cross-linking of OP91 to the monomeric form of Derlin-1 in the same system (Figure 4A, lanes 4 and 5; Figure 4B, lane 5). We therefore conclude that either it is only the monomeric form of Derlin-1 that associates with the OP91 fragment, or that the levels of cross-linking product generated with the Derlin-1 dimer are below the levels of detection for the assay used. Whilst we favour the former interpretation, the levels of dimer that we typically recover by cross-linking are low and we can not at present exclude the alternative conclusion. Furthermore, dimer formation may restrict the accessibility of cysteines within Derlin-1 for cross-linking to the

Figure 4. Derlin-1 monomer cross-links to a misassembled opsin fragment in vitro. (A) A 91 amino acid fragment of opsin with a single cysteine at residue 56 (OP91[cys56]) was synthesized using a rabbit reticulocyte lysate system supplemented with semi-permeabilized HT-1080 cells [20]. Membrane associated radiolabelled polypeptides were isolated and treated with either BMH (lanes 2–4) or a DMSO control (lane 1). BMH treatment of the fully integrated fragment resulted in a number of novel adducts, two of which were immunoprecipitated by an antiserum to the SPP [20], and one of which was immunoprecipitated by two distinct antisera to Derlin-1, but not by a non-related control serum (N.R.). The locations of the fully N-glycosylated (OP91.2CHO) and non-glycosylated (OP91) polypeptides are shown. (B) V5-tagged Derlin-1 was transfected into HT-1080 cells and transiently expressed overnight. The cells were then semi-permeabilized and used to supplement a rabbit reticulocyte lysate system as a source of ER membrane for the integration of the OP91[cys56] fragment described above. BMH-specific adducts were then immunoprecipitated with antisera specific for the SPP, Derlin-1 and the V5 epitope tag.
OP91 fragment via disulphide bonding and/or conformational change of the Derlin-1 monomeric subunits during dimerisation.

Discussion

Our observation that Derlin-1 forms homodimers supports and extends previous studies [8,9]. However, we now show that the level of Derlin-1 homodimer present in the ER membrane is modulated in response to stress. Three well-characterised chemical inducers of ER stress reduced Derlin-1 dimerisation, although XBP-1 splicing was not required for a reduction in Derlin-1 dimer formation. Presumably other factors implicated in ER stress response pathways [29] may also influence the oligomeric status of Derlin-1.

Our identification of a cross-linking product between a misfolded opsin fragment and Derlin-1 provides the first evidence of a US11-independent and direct interaction between Derlin-1 and ERAD substrates [5,6,10] suggesting that Derlin-1 can contribute to the ER associated degradation of endogenous substrates [7,13]. Our data suggest that only monomeric Derlin-1 is associated with this misassembled membrane protein fragment representing a potential ERAD substrate, although we can not formally exclude the dimeric form of Derlin-1 is cross-linked but the resulting adduct is below the level of detection. Assuming that only the monomer of Derlin-1 does interact with ERAD substrates, we envisage a system where two populations of Derlin-1 exist: An active form that we observe as the monomer and a second form that we detect as a dimer. On the basis of our own analysis and that of others [8,9], we believe that chemical cross-linking underestimates the proportion of Derlin-1 that is present in the ER membrane as a homodimer. In this light, we propose that the dimeric form could represent a reserve pool of Derlin-1 that can be made available to increase the cell’s ERAD capacity. The conversion from dimer to monomer may simply result from the sequestration of monomeric Derlin-1 by an increase in misfolded ERAD substrates caused by the various chemical treatments that we have employed. Alternatively, the oligomeric status of Derlin-1 may be modulated by ER stress per se. Since ER stress decreases the level of Derlin-1 dimers present in the ER membrane, we conclude it is most unlikely that a simple homo-oligomer of Derlin-1 acts as the retrotranslocation channel. Rather, we suggest that the released Derlin-1 monomers contribute to the cells ERAD capacity by associating with other components of the ERAD pathway such as Hrd1 and p97 to form large heteromeric complexes that mediate retrotranslocation [5–10]. Our future efforts will seek to clarify the precise function of Derlin-1 and its homologues in relation to the ERAD process.

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