SM-protein-controlled ER-associated degradation discriminates between different SNAREs

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Endoplasmic reticulum (ER)-associated degradation (ERAD) is a specialized activity of the ubiquitin–proteasome system that is involved in clearing the ER of aberrant proteins and regulating the levels of specific ER-resident proteins. Here we show that the yeast ER-SNARE Ufe1, a syntaxin (Qa-SNARE) involved in ER membrane fusion and retrograde transport from the Golgi to the ER, is prone to degradation by an ERAD-like mechanism. Notably, Ufe1 is protected against degradation through binding to Sly1, a known SNARE regulator of the Sec1–Munc18 (SM) protein family. This mechanism is specific for Ufe1, as the stability of another Sly1 partner, the Golgi Qa-SNARE Sed5, is not influenced by Sly1 interaction. Thus, our findings identify Sly1 as a discriminating regulator of SNARE levels and indicate that Sly1-controlled ERAD might regulate the balance between different Qa-SNARE proteins.

Keywords: ERAD; SM proteins; SNARE; ubiquitin; Ufe1

EMBO reports (2007) 8, 1176–1182. doi:10.1038/sj.embor.7401105

INTRODUCTION

Membrane fusion and vesicular transport is crucially controlled by SNARE proteins, which are typically single spanning transmembrane proteins (Jahn & Scheller, 2006). In *Saccharomyces cerevisiae*, the syntaxin (Qa-SNARE) Ufe1 controls homotypic membrane fusion of endoplasmic reticulum (ER) membranes and retrograde transport of coatamer (COPI)-coated vesicles from the *cis*-Golgi to the ER (Downing & Storms, 1996; Lewis *et al*, 1997; Patel *et al*, 1998). By contrast, the related Qa-SNARE Sed5 mediates anterograde transport of COPII-coated vesicles from the ER to the Golgi, and also the fusion of Golgi membranes (Hardwick & Pelham, 1992; Peng & Gallwitz, 2002). For these different functions, Ufe1 and Sed5 collaborate with a specific set of cognate SNAREs of three other SNARE classes—Qb-, Qc- and R-SNARE—to form a tetrameric SNARE bundle (Jahn &

Scheller, 2006). SNARE pairing seems to be guided by proteins of the Sec1–Munc18 (SM) family, but how these proteins direct protein transport is largely unknown (Toonen & Verhage, 2003).

Accumulating evidence indicates that ubiquitin (Ub)–proteasome-dependent ER-associated degradation (ERAD) has a prominent role as regulator of several membrane-related functions (Meusser *et al*, 2005). Notable examples include regulated degradation of the ER-embedded enzymes HMG-CoA reductase, which regulates cholesterol levels, and Ole1, the Δ^9 -fatty acid desaturase from yeast, which supplies cells with unsaturated fatty acids (Braun *et al*, 2002; Meusser *et al*, 2005).

Here, we show that Ufe1 is prone to ubiquitylation and degradation by an ERAD-like mechanism. We found that Ufe1 degradation is negatively controlled by direct binding to the SM protein Sly1. Remarkably, although Sly1 binds to the Golgi syntaxin Sed5 in an analogous manner, it does not regulate Sed5 stability. Thus, our findings underscore the importance of ERAD for controlling membrane function and indicate that the SM protein Sly1 might control the balance between functionally opposing SNARE proteins.

RESULTS

The ER-SNARE Ufe1 is ubiquitylated

Previous studies have indicated that Ufe1, a Qa-SNARE that is embedded in the membrane by its carboxy-terminal tail, is short-lived but only in cells defective in protein kinase-C (Lin *et al*, 2001). However, as no relevant phosphorylation target for this process could be identified, the mechanism of controlled Ufe1 degradation remained unsolved.

When we tested whether Ufe1 is ubiquitylated *in vivo*, we found that it exists at a steady state predominantly in a non-modified form (Fig 1A, left panel). However, when we over-expressed either Ub or a larger, amino-terminally Myc-tagged ubiquitin (^{Myc}Ub) variant, we observed additional slower migrating species, which corresponded to the predicted Ub- or ^{Myc}Ub-modified forms, respectively (Fig 1A, right panel). To extend these findings, we expressed C-terminally haemagglutinin (HA)-epitope-tagged Ufe1 (Ufe1^{6HA}) in Δ ufe1 cells overexpressing either Ub or ^{Myc}Ub and then immunoprecipitated anti-HA-reactive proteins from lysates. Western blot analysis using HA- or Ufe1-specific antibodies identified a characteristic evenly spaced protein ladder, indicating that a fraction of Ufe1 is most likely modified by a

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Received 22 June 2007; revised 17 September 2007; accepted 21 September 2007; published online 9 November 2007

Fig 1 | The ER-SNARE Ufe1 is ubiquitylated *in vivo*. (A) Anti-Ufe1 immunoblots of extracts from cells expressing Ufe1 or a carboxy-terminally epitope-tagged Ufe1 form (Ufe1^{6HA}; left panel), or from cells coexpressing ubiquitin or an amino-terminally epitope-tagged ubiquitin (MycUb; right panel). UFE1 (UFE1^{6HA}) was expressed from its chromosomal locus. The positions of ubiquitin- (Ub-) and MycUb-modified Ufe1 forms are indicated by arrowheads. The asterisk denotes an unrelated crossreacting protein. (B) Immunoprecipitation of C-terminally HA-tagged Ufe1 (Ufe1^{6HA}) under denaturing conditions. WT and tagged Ufe1 proteins were expressed in $\Delta ufe1$ cells at endogenous levels in combination with overexpressed Ub or MycUb. The input and the precipitated material were analysed by western blots using HA- (left panel) or Ufe1-specific antibodies (right panel). The positions of unmodified Ufe1 (Ufe1^{6HA}) and ubiquitin-modified Ufe1 forms (Ub-Ufe1) are indicated. ER, endoplasmic reticulum; HA, haemagglutinin; Ub, ubiquitin; WT, wild type.

polyubiquitin chain (Fig 1B). Notably, Ufe1 ubiquitylation was enhanced when we used a MycUb-expressing strain, which is consistent with the observation that conjugation with MycUb can slow down proteasomal degradation (Ellison & Hochstrasser, 1991).

Sly1 is crucial for Ufe1 but not Sed5 stability

To investigate the turnover of endogenously expressed Ufe1, we performed an expression shut-off experiment by monitoring Ufe1 protein levels after the addition of the translation inhibitor cycloheximide (CHX) to the growth medium. In agreement with the observed low level of polyubiquitylation, Ufe1—and Ufe1^{6HA}—was stable at 23 °C, but moderately short-lived at 37 °C (Fig 2A,B). However, when we repeated this experiment in a temperature-sensitive *sly1-1* mutant (Dascher *et al*, 1991; Cao *et al*, 1998; Kosodo *et al*, 2002), Ufe1 degradation was significantly accelerated at 37 °C, the restrictive temperature of this mutant allele. As Sly1 is a direct interactor of Ufe1, this finding

indicated that Sly1 might control Ufe1 stability through direct physical interaction.

Sly1 is also known to bind to the SNARE Sed5, which is implicated in anterograde transport from the ER to the Golgi. As Sed5 belongs to the same SNARE family as Ufe1 and interacts with Sly1 in an analogous manner (Toonen & Verhage, 2003; Jahn & Scheller, 2006), we asked next whether Sed5 stability is equally affected by the *sly1-1* mutation. However, we observed that under the same conditions Sed5 levels remained constant in the *sly1-1* mutant (Fig 2B), indicating that Sly1 discriminates between the two related SNAREs.

Ufe1 stability is linked to interaction with Sly1

Sly1 carrying an R266K substitution expressed from the *sly1-1* mutant allele fails to interact with Sed5 at the non-permissive temperature (Kosodo *et al*, 2002). Mutation studies indicated that a crucial determinant within the Qa-SNAREs is a conserved short N-terminal stretch, in which a conserved phenylalanine residue (F10 in Sed5 and F9 in Ufe1) contacts a hydrophobic pocket of Sly1 (Bracher & Weissenhorn, 2002; Yamaguchi *et al*, 2002; Peng & Gallwitz, 2004). However, as the amino acid altered by the *sly1-1* allele lies outside this pocket, the observed defect in SNARE binding might be caused by a structural defect of this mutant protein.

To investigate whether Ufe1 stability is linked directly to Sly1 interaction, we introduced mutations that affected the Ufe1–Sly1 interaction specifically either in the N-terminal, Sly1-interacting domain of Ufe1 (Ufe1^{F9A}) or, reciprocally, in the SNARE-binding pocket of Sly1 (Sly1^{L137R} or Sly1^{V156P}). To confirm the anticipated interaction defects, we performed two-hybrid assays using a soluble Ufe1 variant that lacked the transmembrane (TM) domain of the SNARE (UFE1^{ΔTM}). Indeed, analogous to Sed5 (Peng & Gallwitz, 2004), Ufe1 failed to interact with pocket mutant variants of Sly1 (and the *Sly1-1* protein; Fig 3A); the reciprocal Ufe1 mutant protein (Ufe1^{F9A-ΔTM}) also failed to interact with wild-type (WT) Sly1 in this assay.

Remarkably, when we examined the stability of endogenously expressed Ufe1 in these strains—*sly1-1*, *sly1*^{V156P}, *sly1*^{L137R}—we observed rapid Ufe1 degradation in all mutant backgrounds (Fig 3B, upper panel). By contrast, WT and mutant Sly1 proteins were metabolically stable in this assay, indicating that defective Ufe1–Sly1 interaction does not trigger Sly1 instability.

Next, in a reciprocal experiment, we investigated whether the Ufe1 mutant protein defective in Sly1 interaction (Ufe1^{F9A}) is also short-lived. However, as this mutant protein cannot sustain viability of a *ufe1* deletion strain (data not shown), we addressed this question by coexpressing Ufe1^{F9A} as a C-terminally HA-tagged form (Ufe1^{F9A-6HA}) in WT cells. Indeed, this variant was short-lived *in vivo* (Fig 3B, lower panel), indicating that abrogating Sly1–Ufe1 interaction by whichever means results in Ufe1 degradation.

Ufe1 degradation is mediated by ERAD

Ufe1 is a SNARE of ER membranes, and thus we wondered whether Ufe1 degradation is mediated by ERAD. This pathway typically involves the ER-bound Ub-conjugating enzymes Ubc6 and/or Ubc7 (Sommer & Jentsch, 1993; Biederer *et al*, 1997) and the chaperone-like enzyme Cdc48, which is thought to participate in membrane extraction (Meusser *et al*, 2005). We introduced the corresponding mutations into the *sly1-1* background and asked whether they affect Ufe1 degradation. Degradation of endogenous

Fig 2 | Ufe1 but not Sed5 is unstable in the *sly1-1* mutant. (A) CHX-chase experiment with WT (upper panel) and *sly1-1* cells (lower panel). Cells were grown in YPD to an OD₆₀₀ of 0.5 at 23 °C and maintained at this temperature (left) or shifted up to 37 °C after the addition of CHX (right). At each time point, the cellular level of Ufe1 was analysed by anti-Ufe1 immunoblots. As a control, the blots were re-probed with an antibody against the stable ER membrane protein Dpm1. The asterisk indicates a crossreactive band. The graph shows the quantification of the immunoblot signals from the Ufe1 decay (time point zero was set as 100%). (B) Similar to (A) but with strains expressing HA-tagged Ufe1 (Ufe1^{6HA}). Extracts were probed with HA (for tagged Ufe1), Sed5 or Dpm1 antibodies. CHX, cycloheximide; ER, endoplasmic reticulum; HA, haemagglutinin; OD, optical density; WT, wild type.

Ufe1 was indeed proteasome dependent and not mediated by the lysosome, as indicated by a robust stabilization in the conditional proteasomal mutant *pre1-1* (Heinemeyer *et al*, 1991), but not in *Δpep4*, a mutant defective in lysosomal degradation (Fig 4A). Furthermore, Ufe1 degradation in the *sly1-1* background was significantly reduced in strains deficient in Ubc6 and Ubc7 (*Δubc6 Δubc7*), as well as in temperature-sensitive mutants of Cdc48 (*cdc48-6*) or its substrate-recruiting cofactor Ufd1 (*ufd1-2*; Fig 4A).

Conversely, we exogenously expressed the Sly1-interaction-defective Ufe1 (*ufe1^{F9A-6HA}*) variant (in addition to endogenously expressed WT Ufe1; see above) in the same set of strains and monitored its turnover. Indeed, the tested ERAD mutations also caused a stabilization of the substrate in this assay, although only partly in some mutants (Fig 4B). Apparently, the lysosome also contributed to its degradation, suggesting that a small fraction of the mutant Ufe1 variant is routed to the lysosome. Nonetheless, the above findings are consistent with the model that ERAD is the main reason for the observed instability of Ufe1 triggered by defective Ufe1–Sly1 interaction.

Sly1–Ufe1 imbalance is linked to Ufe1 degradation

Next, we investigated whether an imbalance between Ufe1 and Sly1 triggers Ufe1 degradation. We experimentally altered

the Sly1–Ufe1 ratio in cells and found that overexpressed Ufe1 was short-lived (Fig 5A). Intriguingly, however, simultaneous overexpression of Sly1 (Sly1^{6HA}) strongly stabilized overexpressed Ufe1, which thereby accumulated to high levels. We also gradually altered SNARE levels by expressing *UFE1* from its genomic locus, a low-copy-number or a high-copy-number plasmid (all driven by the relatively weak *UFE1* promoter), or even from a high-copy-number plasmid driven by the strong *GAL1-10* promoter. Remarkably, Ufe1 levels accumulated to high steady-state levels only in those cells that also overexpressed Sly1 (Fig 5B).

Importance of Sly1–SNARE and Ufe1–Sed5 balances

The aforementioned data indicate that one function of Sly1 is to protect Ufe1 against degradation by ERAD. We noticed that a double mutant of *sly1-1* with *pre1-1* (defective in proteasomal degradation) is inviable at temperatures at which the single mutants can grow (supplementary Fig S1 online); therefore, we wondered whether misregulated Ufe1 levels are detrimental to growth. Indeed, strong overexpression of Ufe1 is toxic for cells, which is, however, partly relieved by simultaneous overexpression of Sly1 to high levels (which is barely toxic on its own; Fig 6A). This indicates that it is not the high level of Ufe1 as such but the imbalance to Sly1 that is deleterious for cells. Remarkably,

Fig 3 | Loss of Ufe1–Sly1 interaction results in degradation of Ufe1. (A) Two-hybrid interactions between WT or mutant forms of Sly1 and Ufe1 fused to the activating domain (AD) or DNA-binding domain (BD) of Gal4. Transformants were spotted onto control (co) or media plates without histidine (–his) and incubated for 2–3 days. (B) Upper panel: CHX-chase experiments with $\Delta sly1$ cells expressing epitope-tagged proteins of either WT *SLY1* or different *sly1* mutants at endogenous level. The experimental set-up was essentially as described in Fig 2. Protein stability of Ufe1 and the respective Sly1 variant was monitored by anti-Ufe1 (left) and anti-Myc- or anti-HA immunoblots (right), respectively. Lower left panel: anti-HA immunoblot of CHX chase performed at 30 °C with WT cells coexpressing plasmid-borne copies of HA-tagged variants of WT *UFE1* or *ufe1-F9A* at endogenous level. The graph (lower right) shows quantified levels of endogenous Ufe1 and coexpressed Ufe1-F9A^{6HA} in the respective strain backgrounds as indicated. CHX, cycloheximide; HA, haemagglutinin; WT, wild type.

moderate overexpression of Sed5—the alternative SNARE partner of Sly1—restored viability, indicating that not only a Sly1–Ufe1 disparity but also an apparent imbalance between the two SNAREs is detrimental to cellular growth. Notably, strong Sed5 overexpression is also toxic, and either Sly1 or Ufe1 overexpression can alleviate this effect, at least in part (Fig 6B). In agreement with these genetic data, overexpression of Sed5 resulted in a moderate increase of Ufe1 degradation (supplementary Fig S2 online). These data indicate that balanced levels between Ufe1 and Sed5 SNAREs are particularly important for cells.

DISCUSSION

Our finding that SM-controlled ERAD can control SNARE levels has several implications. First, it underscores the role of ERAD in regulating membrane-linked functions and also indicates that potentially unstable ER membrane proteins can be protected against ERAD by an association with partners. More specifically, our data indicate that SM proteins can differentially control SNARE levels.

The role of SM proteins in vesicle fusion events is poorly understood. It has been suggested that they function as safeguards that protect syntaxin SNAREs from mispairing (Toonen & Verhage, 2003). More recent data indicate that they might also protect SNAREs against degradation. For example, deletion of the murine gene for the SM protein Munc18-1 causes a reduction in the level of syntaxin-1 (Toonen *et al*, 2005). Similarly, the yeast SM protein Vps45 protects the SNARE Tlg2 from degradation (Bryant & James,

2001), which seems to be mediated in part by ERAD (supplementary Fig S3 online). Our finding that the SM protein Sly1 controls only Ufe1 but not Sed5 levels provides a possible *raison d'être* for this protective mechanism. As Ufe1 and Sed5 have largely opposing cellular activities, selective degradation of only one SNARE might tip the balance and facilitate one particular direction of vesicular transport. In this speculative model, relatively high Ufe1 levels might foster retrograde transport, whereas low Ufe1 levels caused by ERAD might perhaps stimulate anterograde transport. Indeed, our coexpression experiments indicate that balancing Ufe1 and Sed5 levels might be crucial for growth. As Sed5 has a higher affinity for Sly1 than Ufe1 (Yamaguchi *et al*, 2002), anterograde transport might thus be favoured under normal growth conditions. However, during sporulation, *UFE1* transcripts are upregulated, whereas *SED5* transcripts concomitantly decrease (Chu *et al*, 1998). This indicates that perhaps Ufe1 can accumulate (fostering retrograde transport) only when Sed5 levels are lowered. It will be interesting to see whether, as a general rule, the various routes of vesicular transport are controlled by regulated degradation of SNAREs. Such a mechanism would allow cells to adapt their transport system to demand, for example, for sporulation, active secretion, cellular growth, apoptosis or cell division.

METHODS

Yeast strains and plasmids. Unless otherwise indicated, all strains are isogenic to DF5 (Braun *et al*, 2002), except for the experiment

Fig 4 | Destabilized Ufe1 is degraded by an ERAD-like pathway. (A) Anti-Ufe1 immunoblots of CHX-chase experiments using WT (control; co) and *sly1-1* mutant cells in which additional mutations ($\Delta ubc6 \Delta ubc7$, $\Delta pep4$, *cdc48-6*, *ufd1-2*, *pre1-1*) were introduced. The experimental set-up was essentially as in Fig 2. Representative experiments are shown and the graphs present the quantification of the Ufe1 decay resulting from 3 to 4 independent data sets; symbols and bars represent mean and s.e. (B) Anti-HA immunoblots of a CHX-chase experiment using WT (control; co) and mutants (same as above) coexpressing HA-tagged proteins of WT *UFE1* or *ufe1-F9A* at endogenous *UFE1* levels. The experimental procedure was similar to (A) except that the strains shown in the upper panel were grown to an OD₆₀₀ of 0.6 at 30 °C and kept at this temperature during the time course, whereas the strains shown in the lower panel were grown to an OD₆₀₀ of 0.4 at 23 °C and then shifted for an additional 2 h to 37 °C before the CHX chase was started. Graphs: mean and s.e. of 3–4 independent data sets ($\Delta pep4$, $n = 2$; error bars are deviation from the mean). CHX, cycloheximide; HA, haemagglutinin; OD, optical density; WT, wild type.

in Fig 6B and supplementary Fig S2 online, for which W303 was used for overexpression of *SED5*. Mutant alleles from other strain backgrounds were introduced into DF5 by repetitive mating and tetrad dissection. Further details are available as the supplementary information online.

Antibodies. For raising Ufe1-specific polyclonal antibodies, His-tagged Ufe1 which lacked the C-terminal SNARE and the transmembrane domain (Ufe1 Δ Sn Δ TM, 237–347 aa) was bacterially expressed (*pQE30*; Qiagen, Hilden, Germany) and

affinity-purified under denaturing conditions. The antiserum obtained was affinity-purified against Ufe1 Δ Sn Δ TM–GST expressed from *pGEX4* (Amersham Pharmacia, Uppsala, Sweden). Antibodies against Sed5 and Tlg2 were gifts from D. Gallwitz and H. Pelham, respectively.

Immunotechniques. For examination of post-translational modifications, protein extracts were prepared under denaturing conditions (Knop *et al*, 1999), and subjected to immunoprecipitation in PBS supplemented with 0.1% Nonidet P-40 and 0.05%

Fig 5 | The ratio between Ufe1 and Sly1 affects Ufe1 stability. (A) Expression shut-off experiment monitoring Ufe1 protein stability when expressed endogenously (left panel) or on overexpression (o/e; right panel) in the absence or presence of additionally overexpressed Sly1^{6HA}. In case of overexpression, less sample was subjected to SDS-polyacrylamide gel electrophoresis to visualize protein decay. (B) Steady-state levels of Ufe1 detected by anti-Ufe1 immunoblots (left panel). *UFE1* was expressed from (1) its genomic locus, (2) a low-copy-number centromeric plasmid, or (3) a high-copy-number 2 μ -based plasmid, in each case driven by the *UFE1* promoter, or (4) from a 2 μ -based plasmid driven by the *GAL1-10* promoter. The panels on the right show the same experimental design except that Sly1 (Sly1^{6HA}) is co-overexpressed from a 2 μ -based plasmid under the control of the *GAL1-10* promoter. Dpm1 levels were used as control. HA, haemagglutinin.

Fig 6 | The ratio between Ufe1, Sed5 and Sly1 affects cellular growth. (A) Cytotoxicity mediated by *UFE1* overexpression is reverted by concomitant overexpression of *SLY1* or *SED5*. Cells overexpressing *UFE1* alone (left), or together with *SLY1* (middle), or *SED5* (right) were adapted to galactose-containing media and then plated in fivefold dilutions on media containing galactose or glucose (as control) and incubated for 2–3 days at 30 °C. All constructs were expressed in the DF5 strain from 2 μ -based high-copy-number plasmids under *pGAL1-10* control (except *SED5*: endogenous promoter). Empty vectors are denoted as ‘–’. (B) Cytotoxicity mediated by *SED5* overexpression is partly reverted by concomitant overexpression of *SLY1* or *UFE1*. Cells overexpressing *SED5* alone (left), or together with *SLY1* (middle), or *UFE1* (right) were grown in liquid synthetic media containing raffinose and then plated in fivefold dilutions on media containing galactose or glucose (as control) and incubated for 2–3 days at 30 °C. All constructs were expressed in the W303 strain from high-copy-number plasmids under *pGAL1-10* control.

SDS. After several wash steps, the bound material was eluted with 1% SDS at 65 °C and analysed by western blotting. Further details are available in the supplementary information online.

Expression shut-off experiments. CHX shut-off experiments were performed essentially as described previously (Braun *et al*, 2002).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank U. Cramer and M. Kost for technical assistance, and S. Mishra for experimental help, A. Buchberger, G. Qin and D. Siepe for

discussions and comments on the manuscript, and K.-U. Fröhlich, D. Gallwitz, M. Hochstrasser, M. Knop, H. Pelham and H.-D. Schmitt for reagents. This study was supported (to S.J.) by the Max Planck Society, Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

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