Transmembrane domain length is responsible for the ability of a plant reticulon to shape endoplasmic reticulum tubules *in vivo*

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Summary

Reticulons are integral endoplasmic reticulum (ER) membrane proteins that have the ability to shape the ER into tubules. It is hypothesised that their unusually long, conserved hydrophobic regions cause reticulons to assume a wedge-like topology that induces membrane curvature. Here we provide proof of this hypothesis. When overexpressed, an *Arabidopsis thaliana* reticulon (RTNLB13) localises to, and induces constrictions in, cortical ER tubules. Ectopic expression of RTNLB13 is sufficient to induce ER tubulation in an Arabidopsis mutant (*pah1pah2*) whose ER membrane is mostly in sheet-like form. By sequential shortening of the four transmembrane domains (TMD) of RTNLB13 we show that the length of the transmembrane regions correlates directly with the ability of RTNLB13 to induce membrane tubulation and to form low-mobility complexes within the ER membrane. We also show that full-length TMDs are necessary for residence of RTNLB13 in the ER.

Introduction

Reticulons are integral ER membrane proteins which are ubiquitous in eukaryotes and have been associated with a wide range of biological functions (Yang and Strittmatter, 2007). In particular they have been shown to contribute to shaping mammalian and yeast tubular ER membranes (Voeltz *et al.*, 2006; Hu *et al.*, 2008; Shibata *et al.*, 2008).

Reticulon-like proteins in plants (named RTNLB; Oertle *et al.*, 2003; Nziengui *et al.*, 2007) belong to rather large gene families, with the Arabidopsis genome encoding 21 isoforms (Nziengui and Schoefs, 2009; Sparkes *et al.*, 2009b). All RTNLBs contain a conserved reticulon homology domain (RHD) that comprises two large hydrophobic segments. Such segments are in some cases subdivided into smaller transmembrane domains, which results in a number of possible transmembrane topologies (Yang and Strittmatter, 2007), including a 'W' topology with both N and C-termini located in the cytosol (Voeltz *et al.*, 2006). We have recently shown that five plant reticulon isoforms (RTNLB1-4 and RTNLB13) appear to assume this 'W' topology, which is likely shared by all other Arabidopsis RTNLB isoforms (Sparkes *et al.*, 2010). Reticulons are enriched in ER tubules and their residence in the ER does not require a C-terminal di-lysine motif, which is nonetheless present on most, but not all, RTN isoforms (Nziengui *et al.*, 2007). ER location likely depends on the formation of low-mobility RTN oligomers in the ER membrane (Shibata *et al.*, 2008; Sparkes *et al.*, 2010). When reticulons are overexpressed, the tubular ER is constricted, resulting in reduced diffusion of soluble proteins within the ER lumen (Hu *et al.*, 2008; Tolley *et al.*, 2008).

The membrane topology of reticulons is hypothesized to be both necessary and sufficient to induce membrane curvature (Zimmerberg and Kozlov, 2006; Shibata *et al.*, 2008), with the unusual length of their hydrophobic segments being responsible both for the wedge-shape of the proteins and for their capacity to oligomerise (Shibata *et al.*, 2009). Here we provide

proof of this hypothesis *in vivo* by showing that ectopic expression of Arabidopsis RTNLB13 can convert ER cisternal membranes into tubules, and that this effect is abolished when its TMDs are shortened to the size predicted for most ER membrane-spanning proteins. We also show that the length of the TMD is essential for the ability of RTNLB13 to form complexes and to reside in the ER membrane.

Results and Discussion

Of the 21 putative Arabidopsis reticulon proteins, RTNLB13 is one of the smallest isoforms, comprising the RHD and relatively short N and C termini (Tolley *et al.*, 2008). RTNLB13 is located in the ER membrane. When expressed transiently in tobacco and Arabidopsis, RTNLB13 can induce constrictions in the lumen of the ER (Tolley *et al.*, 2008; Fig 1, panels B-D). RTNLB13 preferentially partitions in the tubular regions of the ER and is excluded from cisternal regions (Sparkes *et al.*, 2010). The propensity of RTNLB13 to localize into high-curvature membranes depends on the presence of a full length RHD (Sparkes *et al.*, 2010). While this indicates that the shape of RTNLB13 is suited to high-curvature membranes, it does not directly prove whether RTNLB13 itself is sufficient for the formation of plant ER tubules.

To test whether RTNLB13 expression is sufficient to induce ER membrane curvature, thus driving the conversion of ER sheets into tubules, we took advantage of the Arabidopsis double mutant *pah1pah2*, which lacks two isoforms of phosphatidic acid phosphatase (Nakamura *et al.*, 2009). Cells of this mutant produce more phospholipids and the ER architecture is severely affected, existing almost entirely in sheet (cisternal) form (Fig. 1, panels E and I) with very few or no detectable tubules (Eastmond *et al.*, 2010). As the plant

cortical ER, unlike the mammalian ER, is predominantly tubular (Sparkes *et al.*, 2009a; Fig. 1A), this loss of tubulation is all the more striking. While it is not known how the *pah1pah2* mutation affects the behaviour of endogenously expressed reticulons, the predominance of sheets makes the *pah1pah2* ER an ideal and unique *in vivo* system to test the general properties of ectopically expressed reticulons. We therefore transfected leaf cells of either wild-type or *pah1pah2* Arabidopsis plants by particle bombardment with plasmids encoding both YFP-RTNLB13 and the luminal ER marker RFP-HDEL and observed ER morphology by confocal microscopy (Fig. 1). When expressed in wild-type leaf epidermal cells, YFP-RTNLB13 induced ER tubule constrictions very similar, although not as pronounced, to those observed in tobacco leaf cells (Fig. 1, panels B-D; Tolley *et al.*, 2008; Sparkes *et al.*, 2010). In *pah1pah2* cells, expression of YFP-RTNLB13 caused the sheet-like ER membrane to revert in part to a tubular phenotype, which is similar to unperturbed ER (Fig. 1, panels F-H and J-L). This indicates that a surfeit of RTNLB13 is capable of inducing ER membrane remodelling and tubule formation *in vivo*. These results are in agreement with previous findings obtained *in vitro* with proteoliposomes, where reticulon dose inversely correlated with the diameter of the induced tubules (Hu *et al.*, 2008).

Having established that ectopic expression of RTNLB13 is sufficient to induce ER tubulation, we tested the hypothesis that this property depends on the length of its transmembrane domains. In accordance with topology predictions, we have previously shown empirically (through RoGFP2 fusions, bimolecular fluorescence complementation and protease protection) that RTNLB13 N- and C- termini face the cytosol and that its RHD comprises 4 distinct TMDs forming a 'W' topology (Sparkes *et al.*, 2010). Each TMD segment is predicted to be 22-23 residues long (Suppl. Fig. S1). This exceeds the predicted length of 17 residues

for plant ER-located transmembrane helices (Brandizzi *et al.*, 2002; Pedrazzini, 2009). Although the structure of the RHD is not known, it is plausible to envisage that, as the TMDs are longer than the predicted thickness of the ER membrane, they cannot insert at right angles into the membrane. This atypical insertion may contribute to the reticulon's wedge-like shape that results in membrane curvature. We therefore hypothesised that sequential shortening of each of the four TMDs would attenuate, and ultimately abolish, the curvature-inducing capacity of RTNLB13. We tested this by generating four YFP-RTNLB13 mutants (ΔTM1-4) in which the regions encoding the transmembrane helices were shortened to 17 residues by deletion of residues at the TMD ends closest to the luminal side of the ER membrane, starting from the first TMD in ΔTM1 and with all four TMD shortened in ΔTM4 (Suppl. Fig. S1B). We expressed these mutants by agroinfiltration of tobacco epidermal cells (Fig. 2). When full-length YFP-RTNLB13 was expressed, the ER lumen, as highlighted by the ER luminal marker RFP-HDEL, was constricted (Fig 2, A-C; Tolley *et al.*, 2008). Mutants with shortened TMDs lost the ability to induce the constriction phenotype (Fig 2, compare panel B with panels E, H, K, N). When 3 out of 4 TMD were shortened, the localisation of ΔTM3 overlapped exactly with normal, unconstricted ER tubules (J-L). When all four TMD were shortened (ΔTM4), cisternal sheets also appeared in the ER network, with YFP-RTNLB13-ΔTM4 being evenly spread over both sheets and tubules (M-O). This is in contrast with the tubule-restricted localisation of wild-type RTNLB13 (Sparkes *et al.*, 2010). A similar phenotype has been previously reported on overexpression of ER membrane proteins such as GFP-calnexin transmembrane domain (Runions *et al.*, 2006).

We then tested if the mutants that have lost the ability to produce tubule constrictions have also lost the capacity to induce *de novo* tubulation, by transiently expressing the TMD

mutants with the most severe loss of function (ΔTM3 and ΔTM4) into *pah1pah2 Arabidopsis* cells (Fig. 3). Occasional tubules were still visible upon ΔTM3 expression, but the ER membrane was mostly in sheet form and the protein partitioned both in sheets and tubules (Fig 3, A-C). When ΔTM4 was expressed, the ER membrane appeared to be in sheet form and was barely distinguishable from the ER of non RTNLB13-transfected *pah1pah2* cells (Fig. 3, D-F, compare with Fig. 1E).

Taken together, these results indicate that the length of the TMDs in RTNLB13 is likely to be responsible for its membrane curvature–inducing properties. When the TMDs are shortened to 17 residues, which is the prevalent TMD size for plant ER membrane proteins (Brandizzi *et al.*, 2002; Pedrazzini, 2009), RTNLB13 loses both the capacity to form tubules and its propensity to inhabit curved membranes. The latter phenotype was also obtained by truncating the RHD (Sparkes *et al.*, 2010). Therefore we conclude that both the complete set of TMD and their 22-23 residue lengths are necessary for the structural properties of this reticulon.

We have recently shown that five plant reticulons isoforms, including RTNLB13, can both homodimerise and form heterodimers with other reticulons, and that an RTNLB13 mutant lacking 2 of its 4 TMDs is still able to homodimerise (Sparkes *et al.*, 2010). It is possible that shortening of its TMDs affects the capacity of RTNLB13 to interact with itself or with other endogenous reticulons, both in the *pah1pah2* mutant and in tobacco epidermal cells. As the exact complement of reticulons in tobacco and Arabidopsis leaves has not yet been defined – and at least 12 distinct isoforms are predicted to be expressed in Arabidopsis leaves (Schmid *et al.*, 2005) - it is not possible to test for individual interactions. We therefore

tested whether the ΔTM mutants can still form an oligomeric complex by measuring their relative mobility within the ER membrane by fluorescence recovery after photobleaching (FRAP). We performed FRAP on the tubular ER of tobacco leaf epidermal cells expressing either YFP-RTNLB13 or YFP-ΔTM4, which has completely lost the capacity to induce tubulation. Figure 4 shows that, while YFP-RTNLB13 fluorescence is severely reduced during photobleaching (Fig. 4A) and only recovers to about 40% of its original intensity with a $T_{1/2}$ of 6.3 s (Fig. 4C), the fluorescence of YFP-ΔTM4 is less drastically reduced, probably due to faster diffusion of unbleached protein into the bleaching region (Fig. 4B), and its recovery is significantly faster, with a $T_{1/2}$ of 2.2 s (Fig. 4C). This indicates that ΔTM4 has a significantly higher mobility within the ER membrane than the wild-type protein. These results mirror those obtained in yeast cells, where an Rtn1p mutant incapable of forming oligomers showed a higher mobility than its wild-type counterpart (Shibata *et al.*, 2008).

Our data therefore provide a link between transmembrane domain length, the capacity to oligomerise and the ability to induce tubule formation of RTNLB13.

We have previously shown that the C-terminal dilysine motif present on RTNLB13 (KKSE) is not necessary for its residence in the ER membrane. Likewise, an intact RHD is not necessary for ER residence of RTNLB1-4 and 13 (Sparkes *et al.*, 2010). Indeed, the presence of either the two N-terminal or the two C-terminal TMD was sufficient to maintain ER location (Sparkes *et al.*, 2010). We therefore hypothesised that ER residence may also depend on TMD length, and that a mutant such as ΔTM4 that has lost the ability to bend the ER membrane and to form complexes would no longer reside there, unless rescued by its C-terminal dilysine motif. To test this, we deleted the KKSE motif from both wild-type and ΔTM4 YFP-tagged RTNLB13 (Fig. 5). Deletion of this motif from both N and C terminal eYFP

fusions to wild-type RTNLB13 did not affect either ER residence or its ability to constrict tubules (Fig. 5, A-C; Sparkes *et al.*, 2010). When KKSE was removed from ΔTM4, however, the protein showed only a minimal labelling of the ER but was instead found in punctate structures (Fig 5, compare panels G-I with D-F). Co-expression with the Golgi marker sialyl transferase signal anchor sequence-RFP (ST-RFP) revealed extensive colocalisation, indicating that YFP-RTNLB13- ΔTM4-ΔKKSE relocates to the Golgi complex (Fig. 5, G-I). Therefore we conclude that the unusual TMD length is also required for the ER localisation of RTNLB13.

Our data show that a 'minimal' plant reticulon such as RTNLB13 is sufficient to induce tubule formation in ER sheets of the *pah1pah2* mutant. The extent of membrane reshaping is not comparable to that observed in the ER of wild-type plants, where the ER membrane is severely constricted. This may depend on the altered lipid composition of the mutant or the distribution of, and interaction with, the endogenous reticulons, which remains to be determined.

Resizing of the transmembrane regions of RTNLB13 affects its capacity to induce membrane curvature, to form complexes within the ER membrane and its very ability to reside in the ER membrane. In the absence of any detailed structural information on reticulon proteins, this indicates a link between TMD length and function. It is possible that the shortening of the TMD results in a change in the topology of RTNLB13. Where two adjacent TMDs have been shortened (for example in mutants ΔTM2 or ΔTM4), their remaining segments may behave as a single TMD, therefore converting the topology of the protein from W to a V. In fact, the TOPCONS topology prediction algorithm (Bernsel *et al.*, 2008) seems to indicate that this is

the case for ΔTM2 - 4 (Fig. S1, panel C). Previously, the C terminus of an RTNLB13 truncation lacking the two C terminal TMDs was shown to reside in the cytosol. In addition, the C terminus of a truncation lacking the last TMD also faced the cytosol, therefore indicating that topology of fusions can alter upon deletion of TMD (Sparkes *et al.*, 2010). TOPCONS prediction of both fusions confirmed the data (data not shown). We believe the experimental verification of this topology change is beyond the scope of this report, but if this is the case, our results indicate that the 'W' topology of the RHD is indeed essential for the interaction and membrane bending properties of RTNLB13. This raises the possibility that other reported topologies for some non-plant reticulons (reviewed in Yang and Strittmatter, 2007) may reflect a non-structural role for these isoforms. Another method of inducing membrane curvature is that of protein scaffolding on the membrane surface (Shibata *et al.*, 2009). However, in this investigation we studied RTNLB13, which is the shortest plant reticulon with N and C terminal cytoplasmic domains of 20 and 47 amino acids respectively. It is therefore unlikely, although not impossible, that these would interact /recruit proteins at the surface which in turn would induce curvature of the ER membrane.

In addition to its loss of membrane-bending capacity, the ΔTM4 mutant of RTNLB13 which lacks the dilysine retrieval signal is no longer retained in the ER. As we recently showed that RTN lacking the two C-terminal TMD and the C-terminal cytosolic domain are still ER located (Sparkes *et al.*, 2010), this indicates that the correct TMD length in both halves of the RHD is required for complex formation, which in turn is likely to afford ER residence.

Our FRAP data and the loss of ER residence of ΔTM4 devoid of its ER retrieval signal indicate that its ability to interact with endogenous reticulons (and possibily with itself) has been severely impaired or abolished. This suggests that homo- or heterotypic protein interactions,

and the subsequent formation of multimeric complexes, depend on the correct TMD length and likely constitute the mechanism that normally retains reticulons in the ER.

It is possible that, during reticulon evolution, conformation-dependent ER membrane localisation, which as we have shown likely depends upon protein-protein interactions within the membrane, may have superseded the need for a canonical ER retrieval signal. Some Arabidopsis reticulon isoforms have lost this signal (Nziengui *et al.*, 2007). Where the dilysine motif persists, however, it can be envisaged that it may act as a safety mechanism to protect as yet unassembled RTN monomers from escaping the ER membrane.

The *pah1pah2* tubulation assay described here provides a novel experimental tool to test reticulon function *in vivo*. Our data also provide a useful framework for elucidating the role of each TMD in the establishment of both homo- and heterotypic reticulon interactions and for the identification of additional factors involved in shaping the plant ER membrane.

Materials and Methods

Recombinant DNA

All RTNLB13 constructs in this study were generated by PCR using YFP-RTNLB13 (Tolley *et al.*, 2008) as a template. Shortening of each TMD region and deletion of the KKSE coding sequence was performed by fusion PCR using the strategy and the primers shown in Fig. S2. All resulting coding sequences were cloned into the XbaI and SacI sites of pVKH18-EN6 (Batoko *et al.*, 2000) and inserted into *Agrobacterium tumefaciens* strain EHA105.

Transient expression

Transient expression in *Nicotiana tabacum* leaf epidermal cells was performed by agroinfiltration as described (Sparkes *et al.*, 2006), using agrobacteria at an OD_{600} of 0.05.

Arabidopsis thaliana leaves were transiently transformed using a Bio-Rad Biolistic® PDS-1000/He Particle Delivery System as described by Nerlich et al., (2007), except that plasmid DNA was coated onto M17 tungsten particles (Bio-Rad).

Confocal microscopy and FRAP analysis

Leaf sectors expressing the fluorescent protein fusions were mounted in water and observed with a 63X (NA 1.4) oil immersion objective on a Leica TCS SP5 confocal microscope. YFP was excited at 514 nm and detected in the 525 to 550 nm range. RFP was excited at 561 nm and detected in the 571 to 638 nm range. Simultaneous detection of YFP and RFP was performed by combining the settings indicated above in the sequential scanning facility of the microscope, as instructed by the manufacturer.

For photobleaching, the tubular ER was magnified using a 10× zoom for clear tubule detection. No drugs were used and stationary regions of the network were targeted for bleaching. Images were acquired every 0.66 s. Three prebleaching frames were acquired and a region of interest 5 μm in diameter bleached at 100% laser intensity for 3 frames. After photobleaching, images were taken at 0.66 s intervals for 20 s. Mean fluorescence intensity within the bleached area was measured during the recovery phase of FRAP experiments using Leica LAS-AF Lite software. Analysis of fluorescence recovery was performed as described by Shibata *et al.* (2008). Briefly, the fluorescence intensity of three regions of interest was measured: the photobleached region (PR), a region outside of the tubular ER network providing overall background fluorescence (BR), and a region within the ER network that was not photobleached to correct for overall observational photobleaching and fluorescence variation (CR). The relative fluorescence intensity (I) for each individual FRAP experiment was background corrected and normalised using the following equation:

$$I=[(PR_t - BR_t)/(PR_{t0} - BR_{t0})*100]x[(100-CR_t/CR_{t0})/100+1]$$

where t_0 values were averaged over the pre-bleach scans. Normalised data for each set of FRAP experiments were fitted in GraphPad Prism 5.0 using the non-linear equation:

$$I = a + (b-a)(1-e^{(-Kt)})$$

where a equals I_0, b is the plateau fluorescence recovery value and K is a rate constant of increase. The half-time ($t_{1/2}$) of fluorescence recovery was derived as ln(2)/K.

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Figure legends

Figure 1. RTNLB13 is sufficient to induce ER tubules *in vivo*.

Wild-type (A-D) or *pah1pah2* (E-L) Arabidopsis leaves were transfected by particle gun bombardment with plasmids encoding the indicated constructs. Transfected leaves were visualised by confocal microscopy. Scale bars, 10 μm

Figure 2. The length of the TMD of RTNLB13 correlates with its membrane bending properties.

Wild-type tobacco leaf epidermal cells were co-infiltrated with *Agrobacterium tumefaciens* carrying the plasmid encoding ER marker RFP-HDEL (green) and either wild-type YFP-RTNLB13 (magenta) or mutants thereof, where the indicated transmembrane domains were shortened from the predicted length of 24 residues to 17 residues. The cartoons at the right show the transmembrane topology of RTNLB13 and indicate (in red) which TMDs have been shortened in the different mutants. Note that the diagram may not reflect the actual topology of the mutants (see Suppl. Fig. S1C). The images shown are representative of 3 independent experiments. Scale bars: A-F, 5 μm; G-O, 10 μm.

Figure 3. Transmembrane domain length of RTNLB13 correlates with its capacity to induce tubules *in vivo*

pah1pah2 arabidopsis leaves were transfected by particle gun bombardment with plasmids encoding the indicated constructs. Transfected leaves were visualised by confocal microscopy. The images shown are representative of 3 independent experiments. Scale bars, 10 μm.

Figure 4. The length of the transmembrane domains of RTNLB13 determines its capacity to form oligomeric complexes in the ER membrane.

Tobacco epidermal cells were transfected with the indicated constructs and subjected to FRAP analysis.

A-B. Representative prebleaching (pre) and 0, 10 and 20 s frames from typical FRAP experiments. The circled areas represent the regions undergoing photobleaching. Scale bars, 2 μm.

C. Fluorescence intensities normalized to prebleach values of FRAP analyses on YFP-RTNLB13 and YFP-ΔTM4 were plotted over time. Error bars indicate ± S.E.; n = 7 independent replicates per construct. The $t_{1/2}$ values for each construct are shown.

Figure 5. The length of the transmembrane domains of RTNLB13 is necessary for its ER localisation.

Tobacco leaf epidermal cells were co-infiltrated with *Agrobacterium tumefaciens* carrying plasmids encoding the indicated constructs. Transfected leaves were visualised by confocal microscopy. The images shown are representative of 3 independent experiments. Scale bars, 10 μm.

Supporting information

Figure S1

A. Diagrammatic representation of the topology of RTNLB13. The transmembrane domains are shown in blue. The regions deleted in the shortened mutant are shown in red. Note that it is not known whether the short loops between TMD1 and 2 and TMD 3 and 4 are actually exposed to the ER lumen.

B. Sequence of RTNLB13. The amino acid residues deleted from the TMD are shown in red.

C. Prediction of transmembrane topology for the proteins described in this study. Prediction was performed using TOPCONS (Bernsel *et al.,* 2008). Note the predicted shift in conformation from 'W' to 'V' in mutants ΔTM2-4.

Figure S2

Diagrammatic representation of the strategy used to generate the RTNLB13 deletion mutants. The primer structure for ΔTM1 is magnified. The dotted lines indicate the target region for deletion. ΔTM1 was generated using YFP-RTNLB13 as a template. Two fragments were generated by PCR using the P1-P5 and P4-P2 primer pairs. The two fragments were then mixed and fused by PCR using the outside primers P1 and P2. ΔTM1 was used as a template for generation of ΔTM2 with P1-P7 and P2-P6. ΔTM2 was the template for generation of ΔTM3 with P1-P9 and P2-P8. ΔTM3 was the template for generation of ΔTM4 with P1-P11 and P2-P10. Deletion of KKSE was performed using primers P1 and P3 using either YFP-RTNLB13 or ΔTM4 as templates.