FEBS Letters 581 (2007) 3356–3362

Reticulon-like proteins in *Arabidopsis thaliana*: Structural organization and ER localization

Hugues Nziengui[1], Karim Bouhidel, David Pillon, Christophe Der, Francis Marty, Benoît Schoefs*

UMR Plante-Microbe-Environnement, INRA 1088/CNRS 5184/Université de Bourgogne, BP 47870, Université de Bourgogne, F-21078 Dijon Cedex, France

Received 8 May 2007; revised 12 June 2007; accepted 12 June 2007

Available online 21 June 2007

Edited by Ulf-Ingo Flügge

Abstract **Reticulons are proteins that have been found predominantly associated with the endoplasmic reticulum in yeast and mammalian cells. While their functions are still poorly understood, recent findings suggest that they participate in the shaping of the tubular endoplamic reticulum (ER). Although reticulon-like proteins have been identified in plants, very little is known about their cellular localization and functions. Here, we characterized the reticulon-like protein family of *Arabidopsis thaliana*. Three subfamilies can be distinguished on the basis of structural organization and sequence homology. We investigated the subcellular localization of two members of the largest subfamily, i.e. AtRTNLB2 and AtRTNLB4, using fluorescent protein tags. The results demonstrate for the first time that plant reticulon-like proteins are associated with the ER. Both AtRTNLB proteins are located in the tubular ER but AtRTNLB4 is also found in the lamellar ER cisternae, and in ER tubules in close association with the chloroplasts. Similarity in protein structure and subcellular localization between AtRTNLB2 and mammalian reticulons suggests that they could assume similar basic functions inside the cell.**

Keywords: Chloroplast; Endoplasmic reticulum; RTLNB; Reticulon; *Arabidopsis thaliana*

1. Introduction

Reticulons (RTNs) were first described as integral membrane proteins of the endoplasmic reticulum (ER) in mammalian neurons [1,2]. RTN genes have now been identified in all eukaryotic genomes studied [3]. RTNs share a 200-amino-acid residue region of sequence similarity at the C-terminus; the reticulon homology domain (RHD), which is responsible for the association to the ER membrane. This region is composed of two hydrophobic regions spaced by an ~60-amino-acid loop (Fig. 1) [4]. RTN membrane topology is thought to be primarily determined by the number of membrane-spanning segments lying within the two hydrophobic regions as illustrated in Fig. 1 [5]. RTNs are predominantly found in the tubular ER where they might act as membrane stabilizers [6]. Mammalian RTNs have been shown to be involved in endocytosis [7], intracellular transport [8,9], and it has been suggested that they play a role in apoptosis, axonal growth and regeneration [5]. Reticulon homologs from nonchordate taxa have been classified into six reticulon-like protein subfamilies (RTNL), including the plant subfamily of RTNLs named RTNLB [3]. Very little is known about the subcellular localization and functions of RTNLBs. Marmagne et al. [10] have identified two RTNLBs from *Arabidopsis thaliana* (AtRTNLB1 and AtRTNLB6) in plasma membrane (PM)-enriched fractions. Hwang and Gelvin [11] have shown that AtRTNLB1 (named VirB2-interacting protein (BTI1)), AtRTNLB2/BTI2 and AtRTNLB4/BTI3 influence the susceptibility to *Agrobacterium tumefaciens*-mediated transformation and interact with VirB2, the major component of the *Agrobacterium* T-pilus. In the same study, the three AtRTLNBs, fused to green fluorescent protein (GFP), have been localized to the periphery of root cells but no association with a specific organelle has been characterized. So far, and in contrast to mammalian RTNs, reticulon-like proteins from plants have not been reported as being localized to the ER.

In the present study, we identified 21 sequences encoding RHD-containing proteins from the *A. thaliana* genome in silico. The overall organization of most AtRTNLBs is reminiscent of those of mammalian RTNs, leading us to suggest that they might share similar functions. Differences within or outside the RHD may however indicate functional differences between members of the AtRTNLB family. We also investigated the subcellular localization of AtRTNLB2 and AtRTLNB4, and observed two different association patterns with ER subcompartments.

2. Materials and methods

2.1. Plant materials and growth conditions

A. thaliana plants (ecotype Columbia) were grown on a 1:1 mixture of soil and compost in a growth chamber under controlled conditions (70% relative humidity, 16 h of light with a photon flux density of

*Corresponding author. Fax: +33 3 80 39 50 66.
E-mail address: benoit.schoefs@u-bourgogne.fr (B. Schoefs).

[1]Present address: Institut für Biologie II, Universität Freiburg, Schänzlestrasse 11, D-79104 Freiburg, Germany.

Abbreviations: At3βHSD/D2, *A. thaliana* 3β-hydroxysteroid dehydrogenase/C-4 decarboxylase 2; BTI, VirB2-interacting protein; CaMV, cauliflower mosaic virus; CTR, C-terminal region; ER, endoplasmic reticulum; EST, expressed sequence tag; GFP, green fluorescent protein; HR, hydrophobic region; NTR, N-terminal region; PM, plasma membrane; RHD, reticulon homology domain; RTN, reticulon; RTNL, reticulon-like protein; RTNLB, reticulon-like protein B; MSS, membrane-spanning segment; YFP, yellow fluorescent protein

0014-5793/$32.00
doi:10.1016/j.febslet.2007.06.032

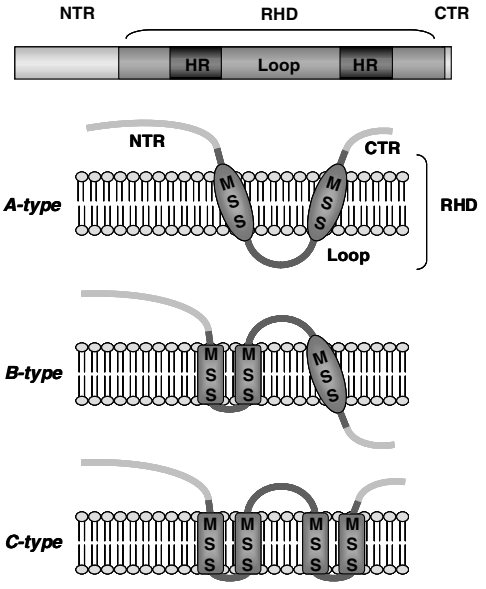

Fig. 1. Structural organization of reticulons and putative membrane topologies. The precise topology of reticulons is not known and the illustrated topologies are based almost exclusively on predictions by computer programs. The N- and C-terminal regions (NTR, CTR) are found on one side or on either of the membrane, depending on the number of membrane-spanning segments (MSS) within each hydrophobic region (HR) of the reticulon homology domain (RHD).

50 μmol m^{-2} s^{-1} at 25 °C and 8 h of dark at 20 °C). Lighting was provided by a combination of fluorescent tubes (Mazda TF36WSA/79) and incandescent lamps (OSRAM 40 W, 230 V, 4 K).

2.2. Bioinformatics analysis

Search for putative RTNLB genes, cDNAs and expressed sequence tag (ESTs) from *A. thaliana* were conducted in TAIR (http://www.arabidopsis.org/) and in Aramemnon databases (http://aramemnon.botanik.uni-koeln.de/). The RHD search was performed with Pfam program (http://pfam.wustl.edu/hmmsearch.shtml). Prediction of membrane-spanning segment (MSS) was made with five different methods (DAS, SOSUI, TMHMM, HMMTOP and TopPred) on ExPASy Proteomics server (http://www.expasy.org/tools/). The number of predicted MSSs varied depending on the method used, so we took into account only MSSs that were identified with at least three different methods. The identification of protein motifs was made with ELM software (http://elm.eu.org/). Sequence alignments were performed by using CLUSTAL W v.1.83 [12] under the following parameter settings: Gonnet 250 as protein weight matrix, 10 and 0.2 as gap opening and gap extension penalties for pairwise and multiple alignments. Aligned sequences were then edited by BioEdit software v.7.0.5 (Carlsbad, CA). The molecular phylogenetic tree was drawn using MEGA version 3.1 [13] as follows: the amino acid sequences were first aligned by CLUSTAL W, then subjected to neighbor-joining tree construction using Poisson correction distance with bootstrap test (500 replicates).

2.3. Plant vector construction

The cDNA clones corresponding to AtRTNLB2 (At4g11220) and AtRTNLB4 (At5g41600) proteins were obtained from RIKEN BioResource Center (http://www.brc.riken.jp/inf/en/). Coding sequences were PCR-amplified using primer pairs GWB2-F (5′-CAATGGCGGATGAACATAAGCATG-3′) and GWB2-R (5′-AATCCTTCTTCTTGTCTTTCAACGG-3′) for AtRTNLB2, GWB4-F (5′-CAACAAAAATGGTGGA-AGACCAC-3′) and GWB4-R (5′-AATCCTTCTTCTTGTTCAGAGC-3) for AtRTNLB4. PCR products were cloned into Gateway entry vector pDONR221 (Invitrogen, Cergy-Pontoise, France), then subcloned into plant transformation vectors pMDC83 [14] and pMDC83Y (a yellow fluorescent protein (YFP)-encoding pMDC83 vector), giving plasmids pRTNLB2:GFP and pRTNLB4:YFP, respectively.

2.4. Plant transformation

The pRTNLB2:GFP and pRTNLB4:YFP plasmids were introduced into *A. tumefaciens* strain C58C1 according to An et al. [15], and the resulting agrobacteria were then used for floral infiltration of *A. thaliana* plants [16]. Infiltrated seeds were surface sterilized and plated onto MS-hygromycin B (20 μg/ml) solid medium. Plates were incubated in the dark at 4 °C for 2 days, exposed to light for 6 h and put back to darkness for 4 days at 22 °C. Hygromycin-resistant seedlings with long hypocotyls were then transferred into soil.

2.5. Protoplast preparation

Protoplasts were prepared from rosette leaves of 3–4-week-old transgenic *A. thaliana* plants as described by Bauer et al. [17].

2.6. Confocal microscopy

Before observation, protoplasts and pieces of leaves were mounted in protoplast medium [17] and water, respectively. When needed, a few drops of the FM-dye FM4-64 (Molecular Probes, Leiden, The Netherlands) at the concentration of 10^{-7} M were added into the mounting medium. Fluorescence observations were conducted with a Leica TCS-SP2-AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany). Excitation wavelengths and emission filters were 488 nm/band-pass 506–538 nm for GFP, 488 nm/band-pass 664–696 nm for FM4-64 and chlorophyll, and 514 nm/band-pass 539–595 nm for YFP. Images were processed using Photoshop 6.0 (Adobe Systems, San Jose, CA).

3. Results and discussion

3.1. The RTNLB protein family in A. thaliana

In order to identify AtRTNLB genes in the *A. thaliana* genome, we blasted the RHD sequence against the TAIR and Aramemnon databases. The search successfully identified 21 genes (Table 1). Fifteen of the 21 gene products are named as AtRTNLB1 to AtRTNLB15 in Aramemnon database. According to this nomenclature, we suggest to name the six other proteins as AtRTNLB16 to AtRTNLB21. At least, one full-length cDNA from TAIR database was associated with all but four AtRTNLBs listed in Table 1. For convenience, we have chosen to report a single splice variant for each AtRTNLB. It is however likely that, similarly to animal RTN genes, a single AtRTNLB gene can give rise to multiple isoforms originating from alternative splicing and/or the use of alternative promoters [3]. Gene expression markers such as ESTs and partial or full-length cDNAs have been found for all but the AtRTNLB7 and AtRTNLB14 genes. The numbers of ESTs indicate that AtRTNLB genes behave differently in terms of expression level and/or range of cell specificity.

Previously identified AtRTNLBs (1–15) display the canonical reticulon organization with (i) a short N-terminal region (NTR: 13–88 aa), and (ii) a C-terminal RHD (Table 1 and [4]). Most of the new RHD-containing proteins (AtRTNLB16–21) show long N-terminal regions (NTR) and/or C-terminal regions (CTR), up to 383 and 85 aa, respectively (Table 1). In Pfam database, less than 1% of all metazoan RHD-protein entries (2/246) displays a CTR. The percentage reaches 11% for RTNLBs (6/55) and 100% for fungi RTN-like proteins (10/10). The functional significance of such differences remains to be elucidated.

The RHD of AtRTNLBs is fairly conserved in length (173–188 aa, Table 1) despite sequence divergence (12–89% identity in pairwise alignments). Asp3 and Trp7 are the only strictly conserved residues among the 21 analyzed RHD sequences (Fig. 2). We used the DAS, SOSUI, TMHMM, HMMTOP, and TopPred methods to characterize the topology of