Calreticulins are not all the same

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Secretory proteins fold and assemble in the endoplasmic reticulum (ER), under the assistance of many folding helpers that also retain newly synthesized polypeptides in this compartment: only upon correct structural maturation are proteins allowed to traffic along the secretory pathway to their destinations. Structurally defective polypeptides that fail correct folding interact more extensively with folding helpers, are retained in the ER, and are often targeted for degradation. Collectively, these events are termed ER quality control (ERQC) (1). A study in this issue of PNAS (2) shows that only one of the three forms of the ER folding helper calreticulin (CRT) of the plant *Arabidopsis thaliana* interacts with a mutated form of BRI1, the plasma membrane leucine-rich-repeat kinase receptor for brassinosteroids, plant-specific hormones playing important roles in plant growth (3). *Arabidopsis* CRT1 and CRT2 are very similar and have homologs in nonplant organisms, but the BRI1-interacting CRT3 seems to be a plant-specific form, with orthologs in higher and lower plant species (4). The important observations by Jin et al. (2) point to plant-specific functional divergence in the CRT family.

CRT is involved in Ca^{2+} storage and the ERQC of N-glycosylated proteins (5). Many secretory proteins are co-translationally N-glycosylated to specific Asn residues by the addition of the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$. Soon after this event, the three Glc residues, which are linearly linked to each other, are sequentially removed by ER glucosidases I (outermost residue) and II (middle and innermost residue). The processing intermediate $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$ is recognized and bound by the soluble lectin CRT or its close relative calnexin, a membrane protein (5). Binding by these ER residents retains intermediates of glycoprotein folding in the ER, prevents unspecific aggregation, and favors the formation of correct disulfide bonds by the action of Erp57, which is in a complex with the lectins (1, 5). Removal of the inmost Glc residue by glucosidase II liberates the newly synthesized glycoprotein, which can, however, be monoglycosylated again by another ER resident, UDPglucose-glycoprotein-glucosyltransferase (UGGT). The cycle of lectin binding and release is interrupted upon correct folding, because UGGT only recognizes non-native struc-

Fig. 1. Interactions of BRI1 with CRT. (Left) Wild-type BRI1 traffics through the Golgi complex to the plasma membrane. This could occur after transient interactions (arrows) with one or more members of the CRT family, which have, however, not been detected to date. (Center) The mutated *bri1-9* strongly interacts with CRT3 but not CRT1/2. This gives a major contribution to retention of the receptor in the ER. (Right) Mutations that cause loss of function of CRT3 (*crt3*) partially suppress the block in traffic, allowing a significant proportion of *bri1-9* to reach the plasma membrane. The schematic CRT structure is based on the model reported by Michalak et al. (5), as follows: oval, N-domain; extended structure, P-domain; square, C-domain of CRT1/2 (light green) or CRT3 (dark green). The two-color square (CRT on the left) indicates uncertain identity of the putative interacting CRT.

tures, thus acting as a sensor of productive folding (5). Another ERQC mechanism, active possibly on every secretory protein and therefore more promiscuous, relies on the activity of the binding protein (BiP), an ER-located member of the heat shock 70 family of molecular chaperones (1). BiP associates to short sequences enriched in hydrophobic amino acids, which are exposed on the surface of folding intermediates and misfolded polypeptides but are masked by correct folding and assembly in most proteins. As is the case of CRT, BiP inhibits aggregation and retains proteins in the ER. The two mechanisms often work in concert. Interestingly, CRT is also able to bind unglycosylated polypeptides and prevent their *in vitro* aggregation, thus acting directly as a chaperone. Also CRT binds sequences enriched in hydrophobic amino acids (6).

ERQC is very efficient but it is not an exception to the rule that no one is perfect. It can fail to recognize defective proteins, leading to the accumulation of harmful structures, or be overzealous in recognition when otherwise functioning proteins are misrecognized as defective

(1). Both types of failure are the bases of important severe human diseases.

Jin et al. (2) have made their exciting discovery during studies that led to the identification of overzealous QC in plants. Some mutated *bri1* alleles encode receptors that are functional but fail to reach the cell surface, thus causing reduced growth and other defective phenotypes associated to the lack of brassinosteroid signaling (7, 8). Jin et al. (2) now show that loss-of-function mutations in CRT3 restore the wild-type phenotype in *bri1-9* plants, acting as suppressors of the *bri1-9* phenotype, but mutations in CRT1/2 have no effect (Fig. 1). *bri1-9* is retained in the ER, but in CRT3-defective plants it correctly reaches the plasma membrane in relevant proportions (ref. 2 and Fig. 1). Co-immunoprecipitation assays detected interactions of *bri1-9*:GFP with CRT3, BiP, and calnexin, but not with CRT1/2 (2, 7). Mutations in CRT3 are not the

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only suppressors of *bri1-9*: the first identified are in UGGT, again pointing to CRT-mediated QC (7). However, loss of function of CRT does not suppress the defective phenotype of all ER-retained *bri1*-mutated alleles, indicating that other folding helpers can also play a key role, depending on the structural defect of the receptor (2, 8).

The *bri1-9* mutation is a Ser-to-Phe change at position 662, an exposed residue conserved among the 25 leucine-rich repeats of the receptor (7). The new highly hydrophobic Phe residue is placed in a segment that already contains a number of hydrophobic amino acids (MMFLDMF₆₆₂YNML) and could create a novel CRT binding site not masked upon folding. But why is this mutation specifically recognized by CRT3? What makes CRT3 different from CRT1/2? All known CRTs are composed of three domains: a globular N-domain, an "extended arm" P-domain, and an acidic C-domain (ref. 5 and Fig. 1). The polypeptide and glycan-binding regions are located in the N- and P-domains. The C-domain is responsible mainly for the Ca²⁺ binding and storage properties of CRT and it is negatively charged in CRT1/2 and the other "classic CRTs" of other organisms, but its C-terminal subdomain is positively charged in CRT3 (4). This is the most striking characteristic of CRT3. Importantly, exchange of the C-terminal portions of CRT1 and CRT3 indicated that the one of CRT3 is necessary and sufficient for the specific action on *bri1-9* (2). The tail of CRT3 may thus be responsible for a more specific role of this isoform in QC, whereas CRT1/2 could be involved mainly in maintaining Ca²⁺ homeostasis (2). This could indicate a specialization of isoforms in plants as opposed to a multifunctional activity of animal CRT (2). A number

of animal CRT features are critical for glycan and polypeptide binding, but none of the key CRT amino acids identified up to now is different in CRT3 or is part of the C-terminal subdomain necessary for its action on *bri1-9* (9, 10). Is wild-type BRI1 a transient substrate of CRT3 or does it switch from transient CRT1/2 binding to strong CRT3 association because of the *bri1-9* mutation (Fig. 1)? Is CRT3 specific for structurally defective polypeptides? This question is still unanswered but the molecular tools are now available for biochemical analysis that can reveal subtle

Loss-of-function mutations in CRT3 restore the wild-type phenotype in *bri1-9* plants.

differences in the recognition properties of CRT isoforms. This can also be tested in cell-free translation-translocation assays, which have already provided evidence for a role of the Glc addition/removal cycle in regulating the assembly of wild-type plant proteins in the ER (11).

Gene expression patterns can also provide clues. CRT3 is limited mainly to leaves, whereas expression of CRT1/2 occurs in many tissues (4). Folding defects in newly synthesized glycoproteins can be induced by treatment with tunicamycin, an inhibitor of N-glycosylation and a potent inducer of the unfolded protein response (UPR), a signaling pathway that up-regulates the expression of ER folding helpers and other regula-

tors of the secretory pathway (12). Microarray analyses detected increased expression of CRT2 and to a lesser extent CRT1 upon 2, 5, or 6 h of tunicamycin treatment of plantlets, but CRT3 seemed unaffected (13, 14). However, in tunicamycin-treated *Arabidopsis* cell cultures CRT3 was induced after 30 min and returned to normal levels after 4 h, with CRT1/2 having an opposite behavior. CRT3 may therefore be specifically subjected to early UPR response (4). UPR can function by two waves of induction, and only the second one seems to be triggered by accumulation of misfolded proteins in the ER (12). Gene coexpression analysis indicates that CRT3 can be grouped with stress resistance genes, whereas CRT1 and CRT2 are coexpressed mainly with other folding helpers (2). The master regulator of systemic-acquired resistance NPR1 positively affects the induction of CRT2/3 and other ER helpers, but not of CRT1, through a *cis* element absent from CRT1 (15). On the whole, these observations point to differences in stress induction of the plant CRT genes, which could reflect different roles in QC.

The distinction between an unfolded protein that will correctly fold and a misfolded one can be very subtle, and their respective destinies can be changed by apparently small differences in thermodynamics properties and alterations in ERQC steps (16). The mutated BRI1 and CRT3 forms will be invaluable in determining the effects of subtle changes on the final outcome of protein folding in the ER of plants and in establishing the patterns of evolution of ERQC in eukaryotes.

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