The Plant Cell, Vol. 10, 119–130, January 1998 © 1998 American Society of Plant Physiologists

The Molecular Chaperone Calnexin Associates with the Vacuolar H^+-ATPase from Oat Seedlings

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Acidification of endomembrane compartments by the vacuolar-type H^+-ATPase (V-ATPase) is central to many cellular processes in eukaryotes, including osmoregulation and protein sorting. The V-ATPase complex consists of a peripheral sector (V_1) and a membrane integral sector (V_o); however, it is unclear how the multimeric enzyme is assembled. A 64-kD polypeptide that had copurified with oat V-ATPase subunits has been identified as calnexin, an integral protein on the endoplasmic reticulum. To determine whether calnexin interacted physically with the V-ATPase, microsomal membranes were Triton X-100 solubilized, and the protein–protein interaction was analyzed by coimmunoprecipitation. Monoclonal antibodies against calnexin precipitated both calnexin and V-ATPase subunits, including A and B and those of 44, 42, 36, 16, and 13 kD. A monoclonal antibody against subunit A precipitated the entire V-ATPase complex as well as calnexin and BiP, an endoplasmic reticulum lumen chaperone. The results support our hypothesis that both calnexin and BiP act as molecular chaperones in the folding and assembly of newly synthesized V_1V_o-ATPases at the endoplasmic reticulum.

INTRODUCTION

Acidification of vacuoles and endomembrane compartments by a vacuolar-type H^+-translocating ATPase (V-ATPase) is essential for many diverse functions in eukaryotes, including the accumulation and storage of ions and metabolites, cytoplasmic homeostasis, osmoregulation, and protein sorting (Klionsky et al., 1990; Sze et al., 1992; Matsuoka et al., 1997). The pump is a multimeric complex with seven to 13 different subunits; however, it is unclear how the enzyme complex is synthesized and assembled to form an active H^+-ATPase. The peripheral V_1 complex of five to eight different subunits contains the catalytic subunit A and regulatory subunit B, both of which bind ATP. The membrane integral V_o sector is a complex of two to five subunits that form the proton-conducting channel (Kane and Stevens, 1992; Sze, et al., 1992; Hirata et al., 1997).

Analysis of yeast *vma* (for vacuolar membrane ATPase) mutants has suggested that the V_1 and V_o sectors are synthesized independently (Kane and Stevens, 1992). A V_1 complex is synthesized and assembled in the cytosol in *vma3* mutants lacking the membrane integral 16-kD subunit (Doherty and Kane, 1993; Tomashek et al., 1996). Furthermore, a membrane integral V_o complex is synthesized and assembled in mutants defective in any single V_1 subunit. An assembled V_o from *vma2* mutants can attach to a wild-type V_1 to form an active ATPase (Parra and Kane, 1996). The results support a model that an assembled V_1 complex attaches to the V_o sector either at the endoplasmic reticulum (ER) or at some later stage of the secretory pathway (Kane and Stevens, 1992).

We and others have provided direct evidence for the presence of V_1 subunits at the ER from plant tissues. Immunogold labeling of oat root tips with a monoclonal antibody (MAb) against the peripheral subunit B showed that gold particles decorated the ER as well as provacuoles (Herman et al., 1994). The results are consistent with the localization of V_1 and V_o subunits on the ER and on vacuolar membranes fractionated by using a sucrose gradient. Oberbeck et al. (1994) showed that purified membrane fractions of the ER, Golgi apparatus, and clathrin-coated vesicles from maize roots reacted positively in immunoblots with antibodies against both peripheral and integral subunits of the V-ATPase. These results support a model in which the V_1 is assembled to the V_o at the ER.

Recent studies with yeast and animal systems show that the folding and oligomerization of membrane and secretory protein complexes depend on a set of proteins in the ER called molecular chaperones. Available evidence suggests that chaperones function primarily by preventing premature folding and aggregation of intermediates, thus allowing productive

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folding and assembly to proceed more efficiently. Several ER chaperones, including BiP (Hammond and Helenius, 1994), calreticulin (Hebert et al., 1996), GRP94 (Melnick et al., 1994), and calnexin (Hammond and Helenius, 1994; Williams, 1995), have been discovered, and the specific role of each is being defined. For example, BiP, a lumenal protein, is required for the translocation of soluble proteins into the ER (Nyugen et al., 1991); it also participates with other chaperones in the folding and assembly of membrane proteins that transit from the ER to their final destination (Hammond and Helenius, 1994). The function of calnexin as a potential chaperone initially came from the demonstration that it is transiently associated with the newly synthesized heavy chain of the class I histocompatibility complex at the ER (Degen and Williams, 1991).

Recent findings show that calnexin, an integral protein, binds transiently to a wide array of membrane proteins (Bergeron et al., 1994), including the influenza hemagglutinin (Hebert et al., 1996), the vesicular stomatitis virus (VSV) G glycoprotein (Hammond and Helenius, 1994), and the multidrug resistance P glycoprotein (Loo and Clarke, 1994, 1995), as well as soluble secretory proteins such as thyroglobulin (Kim and Avron, 1995). One function of calnexin is to retain incorrectly or incompletely folded proteins (Williams, 1995). The role of ER chaperones in plants is less well understood. Chaperones, like BiP, play a role in the folding and assembly of newly synthesized seed storage proteins in the ER; however, the biological function of calnexin remains unclear (Boston et al., 1996).

It is not known whether any of the general ER-localized chaperones participate in the folding and assembly of the V-ATPase complex from eukaryotes, including yeast. The assembly of the V-ATPase in yeast depends on three novel ER-associated proteins. Interestingly, the 25-kD Vma12p, the 8-kD Vma21p, and the 21-kD Vma22p proteins are probably chaperones required specifically for the proper assembly of V_o subunits but not other secretory or membrane proteins (Hirata et al., 1993; Hill and Stevens, 1994, 1995). Here, we show by coimmunoprecipitation that both calnexin and BiP are physically associated with a V-ATPase complex that includes both V_1 and V_o subunits. These results provide direct evidence supporting a model in which calnexin and BiP participate in the folding and assembly of the V-ATPase at the ER of eukaryotes.

RESULTS

An Integral Protein on the ER Copurified with a V-ATPase

When a V-ATPase purified by gel filtration was used to generate antibodies in mouse (Ward et al., 1992), several MAbs (MAb 11A1 and MAb 12A2) recognized a 64-kD (roots) or a 67-kD (shoots) polypeptide that surprisingly was not part of the multimeric ATPase (Ward, 1991). Thus, a 64-kD protein had apparently copurified with the V-ATPase from roots (Ward and Sze, 1992), although its identity and function remain unknown. One working idea is that the protein is required for the synthesis, assembly, or targeting of the endomembrane proton pump. To test this idea, we used either roots or shoots of young seedlings because the level and the synthesis of V-ATPase are relatively high in growing tissues (Randall and Sze, 1989).

To determine the subcellular localization of the 64-kD protein, the postmitochondrial supernatant consisting of microsomal membranes was fractionated on an isopycnic sucrose gradient. Both the 64-kD protein and BiP, a 78-kD ER lumen chaperone, equilibrated to a density corresponding to 26 to 33% sucrose (Figure 1A). Subunits A (70 kD) and B (60 kD) of the V-ATPase were distributed at densities ranging from 19 to 33% sucrose (Figure 1B). Vacuolar membrane protein VM23 and the plasma membrane H^+-ATPase equilibrated at 19 to 24% and 36 to 42% sucrose, respectively, on a similar sucrose gradient (Herman et al., 1994). The presence of subunits A and B at 25 to 33% sucrose suggests that part of the V-ATPases colocalized with the 64-kD protein in the ER.

To determine whether the protein was peripheral or integral, membranes were treated with various detergents, high salt, or urea, and the soluble and pellet fractions were probed for the polypeptide by using the MAb 11A1. After we washed membranes with 0.5 M NaCl or 2.5 M urea, the entire immunoreactive 64-kD protein was associated with the pellet fraction, as occurred with the buffer-treated control (Figure 2A, top). In contrast, BiP was detected in the soluble

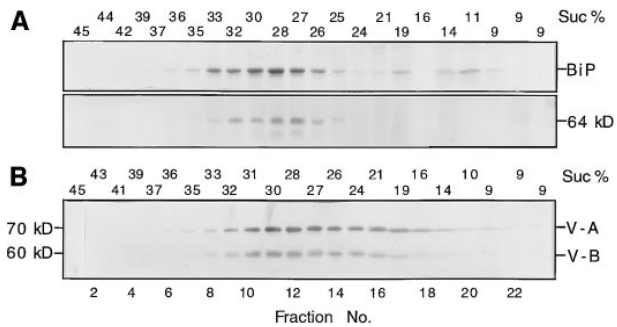

Figure 1. MAb 11A1 Recognizes a 64-kD Polypeptide That Comigrated with an ER Protein.

The postmitochondrial supernatant from oat roots was fractionated on a 15 to 45% linear sucrose (Suc) gradient. The protein in each fraction was analyzed by SDS-PAGE and transferred to an Immobilon-P membrane.

(A) A 64-kD polypeptide comigrates with BiP. Blots were immunostained with MAb 11A1 and polyclonal antibodies against BiP.

(B) V-ATPase subunits partially comigrate with BiP. The blot was immunostained with a mixture of MAbs 7A5 and 2E7 against subunits A (V-A; 70 kD) and B (V-B; 60 kD), respectively.

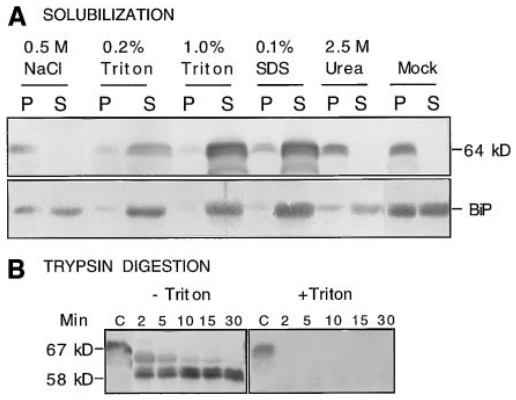

Figure 2. MAb 11A1 Recognizes an Integral Membrane Protein.

(A) Detergents solubilize the protein. Microsomal vesicles from roots were treated with various reagents for 30 min and centrifuged. The soluble (S) and the pellet (P) fractions were analyzed by SDS-PAGE and immunostained with MAb 11A1 (1:100) and polyclonal antibodies to BiP (1:30). Triton, Triton X-100.

(B) Trypsin digests the protein after vesicle disruption. Microsomal membranes from shoots were incubated with trypsin (100 μg/mL) for various times at room temperature with (+) or without (−) 2% Triton X-100. The reaction was stopped and boiled. An aliquot (~20 μg of protein) was analyzed by SDS-PAGE and immunostained with MAb 11A1. C, control membrane.

fraction even after a buffer wash (Mock). The results are consistent with the idea that BiP, as a soluble lumen protein, had escaped from leaky or unsealed vesicles. However, the 64-kD protein appeared in the soluble fraction only after treatment with Triton X-100 or SDS (Figure 2A), indicating that the protein was solubilized from the membrane by detergents and thus is an integral membrane protein.

Vesicles were treated with trypsin to test whether the unidentified protein had been exposed to the cytoplasmic face. In the absence of Triton X-100, trypsin decreased the molecular mass of the unidentified protein by only 9 kD, indicating that a short section of the protein was exposed to the cytoplasmic side (Figure 2B). In the presence of 2% Triton X-100, the protein was undetectable in immunoblots (Figure 2B, right), suggesting that disruption and solubilization of the vesicle by Triton X-100 exposed the polypeptide to degradation by trypsin. These results indicate that most of the molecule faced the lumenal side of the vesicle membrane and was protected from enzyme digestion in the absence of detergent.

Identification of the Integral Protein as Calnexin

To determine whether the unidentified protein was calnexin, cDNA clones encoding calnexin from Arabidopsis were obtained from N. Hoffman (see Huang et al., 1993). Restriction fragments encoding either the N-terminal domain or an internal domain of Arabidopsis calnexin (Figure 3A) were cloned into either a histidine-tagged pET28 or a pMAL-cRI expression vector (Huang et al., 1993), respectively, and the fusion proteins were expressed in *Escherichia coli.* In the presence of isopropyl β-D-thiogalactopyranoside (IPTG), a fusion protein of 66 kD, corresponding to the N-terminal half of calnexin (N-CNX), reacted with a polyclonal antibody against calnexin (Figure 3B, lanes 2 to 4), MAb 11A1 (Figure 3B, lanes 9 to 11), and MAb 12A2 (data not shown). However, a 61-kD fusion protein with an internal region of calnexin (Int-CNX) was not recognized by MAb 11A1 after induction with IPTG (Figure 3C, lanes 10 to 12). The polyclonal antibody previously generated against an internal region of calnexin (Huang et al., 1993) reacted with the

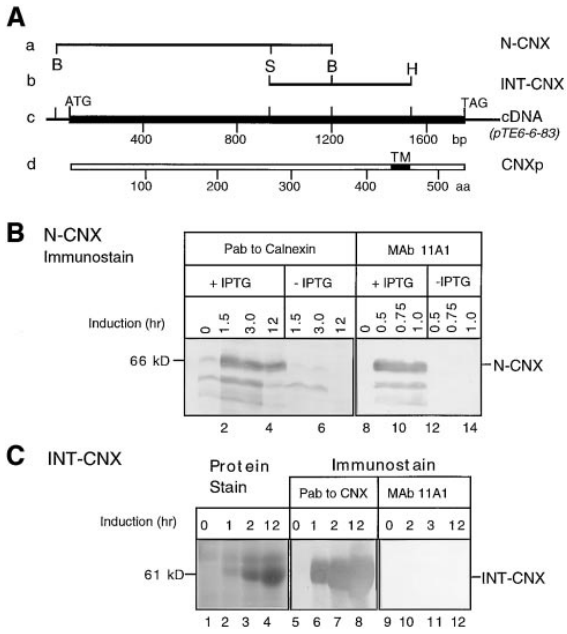

Figure 3. MAb 11A1 Immunoreacts with the N-Terminal Region of Arabidopsis Calnexin.

(A) cDNA fragments used to express two regions of calnexin. A 1161-bp BglII (B) fragment encoding the N-terminal domain (a; N-CNX) or a 615-bp SalI (S)–HindIII (H) fragment corresponding to an internal region of calnexin (b; INT-CNX) was subcloned into expression vectors. The full-length cDNA is shown in (c). The deduced polypeptide of calnexin (d; CNXp) has one putative transmembrane domain (TM). aa, amino acid.

(B) Mab 11A1 recognizes a 66-kD fusion protein expressing the N-CNX. The fusion protein was expressed in *E. coli* with (+) or without (−) IPTG for varying intervals. Total protein was extracted, separated by SDS-PAGE, blotted, and immunostained with either MAb 11A1 (1:100) or polyclonal antibodies (Pab) against Arabidopsis calnexin (1:3000).

(C) MAb 11A1 does not recognize a 61-kD fusion protein expressing INT-CNX. The fusion protein was induced and analyzed as given in **(B)**. Protein staining was with Coomassie blue.

Int-CNX fusion protein (Figure 3C, lanes 6 to 8). MAb 11A1 did not react with the tag, because a Ca-ATPase peptide (Liang et al., 1997) fused to a histidine tag was not immunoreactive (data not shown). Thus, the antibody specifically recognizes an epitope that falls between the N terminus and the middle of the calnexin polypeptide. In addition, the epitope recognized by MAb 11A1 is apparently conserved among plants, including calnexin from oat and from Arabidopsis.

Association of Calnexin with Protein Complexes

If calnexin acts as a chaperone to fold and assemble proteins synthesized in the ER of plant cells as it does in animal cells, it should associate with other proteins. To test this hypothesis, microsomal proteins were solubilized with detergents and then subjected to velocity centrifugation in a 5 to 26% sucrose gradient. When membrane proteins were solubilized with SDS, which disrupts the tertiary and quaternary structure of proteins, calnexin or subunit B of the V-ATPase was detected by immunostaining at the top of the gradient (6 or 7% sucrose), as is expected of monomeric proteins (Figure 4A). When membranes were solubilized with Triton X-100, calnexin and subunit B sedimented to 9 to 13% and 14 to 26% sucrose, respectively (Figure 4B). Because Triton X-100 is a mild nonionic detergent that leaves the tertiary and quaternary structure of the proteins unaltered, the results indicate that subunit B and calnexin are associated with large protein complexes.

Based on the sedimentation pattern of several standard proteins, we estimated that calnexin–protein complexes ranged from 200 to 400 kD. Thyroglobulin (669 kD), apoferritin (443 kD), and β-amylase (200 kD) moved to 23, 15, and 10% sucrose, respectively, in a similar gradient. The migration of subunit B from 14 to 26% sucrose probably represented the separation of V-ATPase complexes ranging from 400 to 700 kD. Because the molecular mass of a purified and active V-ATPase from oat root is 650 kD (Ward and Sze, 1992), these complexes could include partially and fully assembled V-ATPases, as determined by the comigration of V_1 and V_o subunits, including those of 42, 36, and 16 kD (Figure 4C).

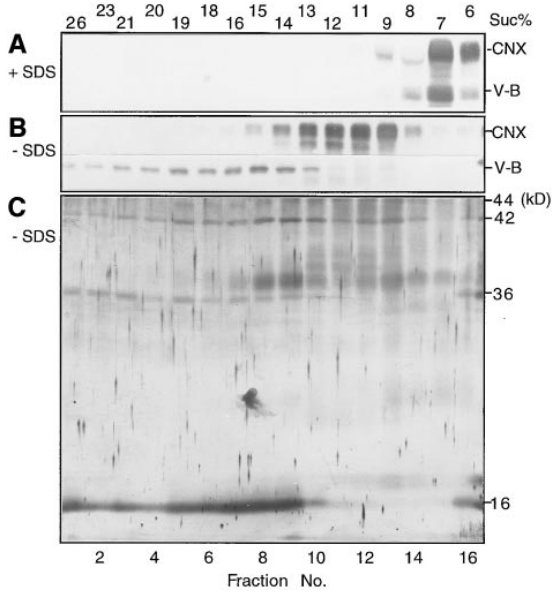

Figure 4. Association of Calnexin with Protein Complexes.

Microsomal protein was solubilized in Triton X-100 with (+) or without (−) SDS and fractionated on a 5 to 26% sucrose (Suc) gradient containing 1% Triton X-100 with or without 0.1% SDS. Fractions were analyzed by SDS-PAGE, blotted, and immunostained.

(A) Sedimentation of monomeric calnexin and subunit B. Protein was solubilized with 2% Triton X-100 and 1% SDS. The blot was stained with a mixture of MAb 11A1 (CNX) and MAb 2E7 to subunit B of V-ATPase (V-B).

(B) Sedimentation of native calnexin and subunit B. Membrane protein was solubilized with 4% Triton X-100. The blot was immunostained with MAb 2E7 (V-B) and MAb 11A1 (CNX).

(C) Sedimentation of V-ATPase subunits. Membrane protein was solubilized in 4% Triton X-100. The blot was immunostained with polyclonal antibodies against the V-ATPase holoenzyme. Thyroglobulin (669 kD), apoferritin (443 kD), and β-amylase (200 kD) migrated to 23, 15, and 10% sucrose, respectively (data not shown). Numbers at right indicate the molecular mass of V-ATPase subunits.

Coimmunoprecipitation of V-ATPase and Calnexin

To determine whether calnexin associated directly with V-ATPase subunits, we tested for protein–protein interactions by using reciprocal immunoprecipitation (Phizicky and Fields, 1995). Either the antibody against calnexin or the antibody against a subunit of the V-ATPase should coimmunoprecipitate the antigen and the associated proteins. An important criterion is that the antibody against calnexin does not recognize V-ATPase subunits and the antibody against a V-ATPase subunit does not recognize calnexin. MAbs 2E7 and 8B6 specifically recognized subunits B and A, respectively, but not any other proteins in immunoblots of total microsomal protein (Figure 5A, lanes 1 and 2). Both MAbs 11A1 and 12A2 specifically recognized calnexin, although the isoforms found in the shoot were apparently larger (67 kD) than the isoforms from the root (64 kD) (Figure 5A, lanes 3 and 4). Despite the striking similarity in the ER-luminal domain of calnexin with calreticulin (Denecke et al., 1995), MAb 11A1 or 12A2 clearly did not recognize the ER lumenal homolog of 55 to 60 kD (Figure 5A).

We tested the specificity of MAb 8B6 by using immunoprecipitation before and after complex dissociation with SDS. Membranes were solubilized with different concentrations of SDS in the presence of 4% Triton X-100. At 1.6% SDS, MAb 8B6 immunoprecipitated a major polypeptide of 70 kD (Figure 5B, lane 8). The identity of the 70-kD polypep-

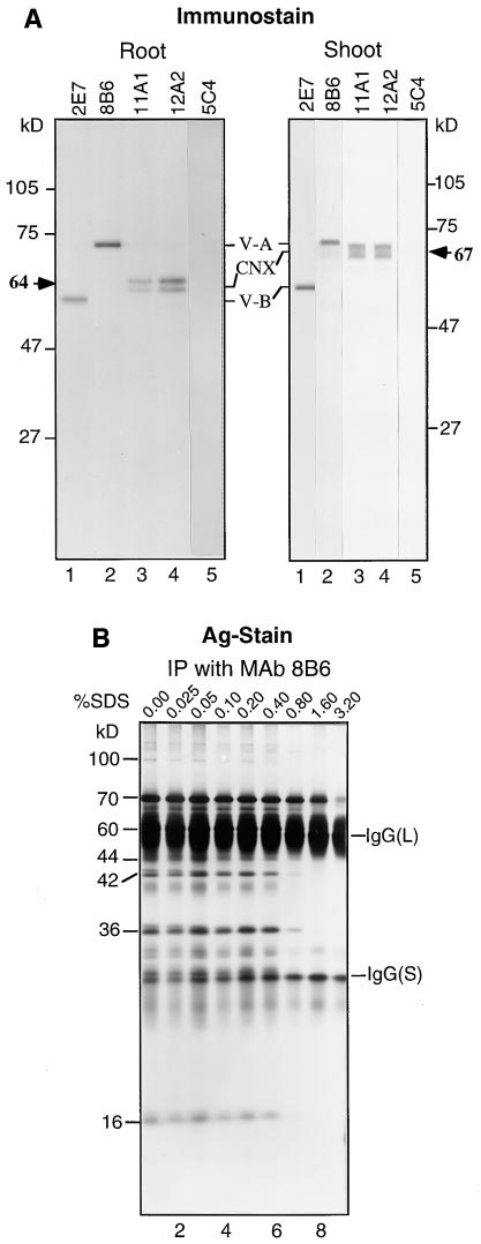

Figure 5. Specificity of MAbs.

(A) MAb 11A1 or Mab 12A2 recognizes calnexin (CNX), whereas MAbs 8B6 and 2E7 recognize V-ATPase subunits A and B (V-A and V-B), respectively. Proteins in microsomal vesicles from shoots (right) and roots (left) were separated by SDS-PAGE on a single-comb slab gel and immunostained with different MAbs using a multichannel blotter. MAb 5C4 was used as a control (lanes 5). Molecular mass standards are indicated at left and right. Arrows indicate calnexin.

(B) MAb 8B6 immunoprecipitates one polypeptide of ~70 kD after SDS treatment. Microsomal protein (1 mg/mL) was solubilized with 4% Triton X-100 and SDS (0 to 3.2%). The solubilized material was immunoprecipitated (IP) with MAb 8B6, and one-half of the precipitated proteins from each treatment were analyzed by SDS-PAGE and stained with silver. IgG(L) and IgG(S) refer to the large and small chains of the IgGs. The molecular masses of the precipitated polypeptides are indicated at the left.

tide as subunit A was verified by immunoblotting (data not shown). Polypeptides of ~55 and ~25 kD corresponded to the heavy and light chains of the immunoglobulins, respectively. Thus, MAb 8B6 specifically recognized subunit A and immunoprecipitated this polypeptide alone when subunit interactions were destroyed by SDS. Similar control experiments were conducted to establish the specificity of MAbs against calnexin when immunoprecipitated (data not shown).

When membrane proteins were solubilized in 4% Triton X-100 alone or with low levels of SDS (<0.4%), MAb 8B6 coimmunoprecipitated several polypeptides (Figure 5B, lanes 1 to 6) that correspond in molecular masses to subunits of the purified V-ATPase (Ward and Sze, 1992). Thus, when protein–protein interactions between subunits are retained after solubilization with the nonionic detergent, MAb 8B6 specifically coimmunoprecipitated a set of polypeptides that form a native complex with subunit A. MAb 5C4, generated at the same time as the MAb to V-ATPase, did not react with any microsomal protein (Figure 5A, lane 5) and therefore was used as a control MAb in all immunoprecipitation experiments (Figures 6A and 6B, lanes 1; Figure 7, lane 2).

In addition to the entire V-ATPase complex, MAb 8B6 against subunit A also precipitated calnexin. The complex immunoprecipitated by MAb 8B6 included subunits A (70 kD) and B (60 kD) and those of 44, 42, and 36 kD from the peripheral V_1 complex as well as the membrane-associated (V_o) 16-kD subunit (Figure 6A, lane 2). Interestingly, a 100-kD polypeptide was part of the complex, although not at stoichiometric levels. The identity of these proteins was further confirmed by the immunoreactivity of MAbs to subunits A and B and with polyclonal antibodies recognizing V-ATPase subunits from 16 to 44 kD (Figure 6B, lane 2). A small amount of calnexin (67 kD) was precipitated consistently by MAb 8B6 and was distinguished from subunit A by immunostaining with MAb 11A1 (Figure 6B, lane 2). Increasing the amount of the antibody above 100 μL per reaction mixture did not signficantly increase the level of calnexin that was precipitated (data not shown). These results suggest that only a small fraction of the V-ATPase complexes is associated with calnexin.

Significantly, a mixture of MAbs 11A1 and 12A2 immunoprecipitated calnexin as well as subunits of the V-ATPase. Although calnexin is the major protein precipitated, V-ATPase subunits that consistently coprecipitated included A and B and those of 42, 36, and 16 kD (Figure 6B, lane 3). Thus, both calnexin and V-ATPase were coimmunoprecipitated by using either antibody, indicating that they were physically

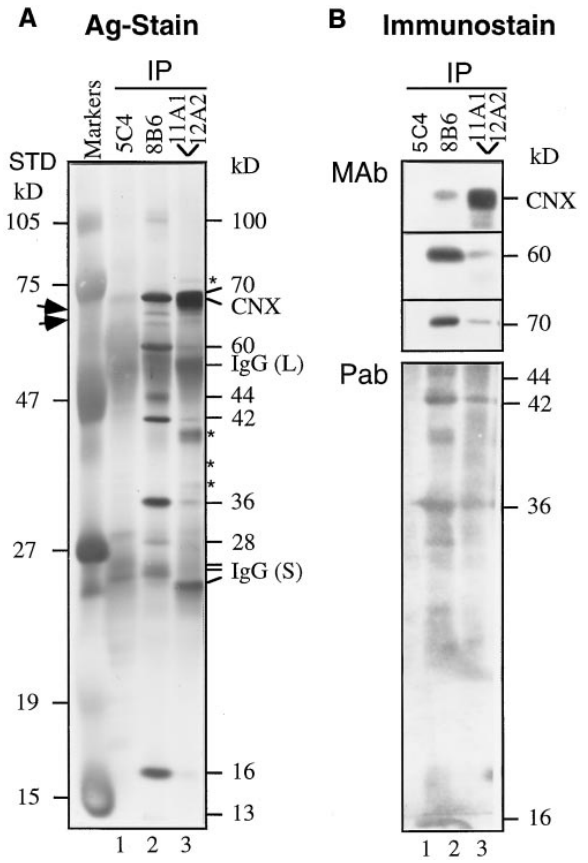

Figure 6. Coimmunoprecipitation of V-ATPase Subunits and Calnexin by Either a MAb against Subunit A or a MAb against Calnexin.

Triton X-100–solubilized microsomal proteins (~1 mg/mL) were immunoprecipitated (IP) with a control, MAb 5C4 (lanes 1), MAb 8B6 (lanes 2), or a mixture of MAbs 11A1 and 12A2 (lanes 3) and separated by SDS-PAGE.

(A) Precipitated proteins stained with silver. Molecular mass markers (STD kD) are indicated at left. Molecular masses of oat V-ATPase subunits are at right. IgG (L) and IgG (S) refer to the large and small chains of the IgGs, respectively; CNX indicates calnexin. Asterisks (right) and arrows (left) indicate additional proteins immunoprecipitated by MAbs 11A1 and 12A2 and by MAb 8B6, respectively.

(B) Identification of proteins by immunostaining. Proteins were transblotted and immunostained with a MAb (top) against calnexin (CNX) and subunit A or B (70 or 60 kD, respectively) or with polyclonal antibodies (Pab) (bottom) against the V-ATPase holoenzyme (1:500). Molecular masses of known V-ATPase subunits from oat are shown at right.

associated with one another. Because a similar concentration of membrane protein was solubilized in parallel reactions and the level of the antibody was adjusted to yield optimal immunoprecipitation, the results also show that at any given time, only a small percentage of total calnexin was bound with the V-ATPase.

Association of Calnexin with the Plasma Membrane H^+-ATPase

To determine whether membrane proteins might interact nonspecifically or were trapped in a detergent micelle, we tested whether calnexin associated with the plasma membrane H^+-ATPase independent of its interaction with the V-ATPase. Interestingly, a MAb against calnexin consistently coimmunoprecipitated a 100-kD polypeptide that reacted with antibodies against the plasma membrane H^+-ATPase (Figure 7, lane 1). Importantly, MAb 8B6 against subunit A immunoprecipitated calnexin but not the plasma membrane H^+-ATPase (Figure 7, lane 3), indicating that the calnexin–V-ATPase complex was separate and independent of the calnexin–plasma membrane H^+-ATPase complex. Thus, the coimmunoprecipitations (Figure 6) did not result from nonspecific associations or aggregration. In addition, these results support the notion that calnexin is also involved in the folding and assembly of the plasma membrane H^+-ATPase at the ER before the pump is sorted to the plasma membrane.

Association of BiP with the V-ATPase

To test whether a soluble ER chaperone interacted with the V-ATPase, microsomal membrane was separated from the cytosolic fraction by centrifugation of the postmitochondrial supernatant. From the detergent-solubilized microsomal proteins, MAb 8B6 precipitated the entire V_1V_o complex, including the integral 16-kD subunit and a 100-kD protein (Figure 8A, lane 1). In contrast, the same MAb precipitated mainly V_1 subunits from the cytosolic fraction, indicating the presence

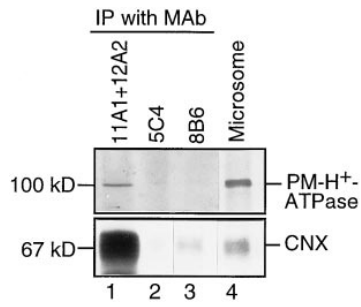

Figure 7. Coprecipitation of the Plasma Membrane H^+-ATPase with Calnexin.

Triton X-100–solubilized proteins of microsomal membranes were immunoprecipitated (IP) by MAb 11A1 plus 12A2 (lane 1), MAb 5C4 (lane 2; control), or MAb 8B6 (lane 3). Proteins were separated by SDS-PAGE, transferred to an Immobilon-P membrane, and immunostained with either polyclonal antibodies against plasma membrane (PM) H^+-ATPase (top) or MAb 11A1 (CNX; bottom). Lane 4 is the control blot of total microsomal protein.

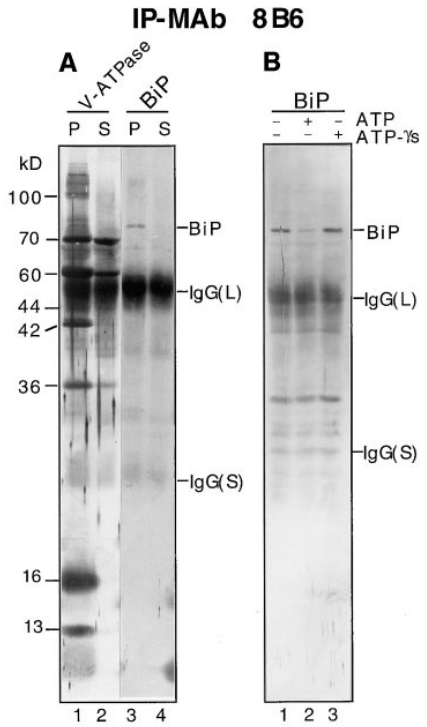

Figure 8. BiP Associated with V-ATPase.

(A) Coprecipitation of BiP with V-ATPase by a MAb against subunit A. The postmitochondrial supernatant (3 mg of protein in 2 mL) was centrifuged at 150,000*g*. One milliliter of the resulting supernatant (S) was immunoprecipitated (IP) with MAb 8B6. The pellet (P) was first solubilized with 4% Triton X-100 (2 mL), and 1 mL of the solubilized material was immunoprecipitated. Half of the precipitated proteins were analyzed by 12.5% SDS-PAGE, blotted, and immunostained with either polyclonal antibodies against the V-ATPase holoenzyme (lanes 1 and 2) or polyclonal antibodies against BiP (lanes 3 and 4). The molecular masses of V-ATPase subunits are shown at left. IgG(L) and IgG(S), large and small chains of the IgGs, respectively.
(B) ATP-induced dissociation of BiP. Microsomal proteins were Triton X-100 solubilized, and 1 mL of the solubilized material was incubated for 1 hr with buffer (lane 1), 1 mM ATP (lane 2), or 1 mM ATP-γ-S (lane 3) in the presence of MAb 8B6. The immune complexes were then treated with PAM and precipitated. Half of each precipitated material was analyzed by SDS-PAGE, blotted, and stained with polyclonal antibodies against BiP.

of partially and fully assembled V_1 subcomplexes (Figure 8A, lane 2). Importantly, BiP was coprecipitated with the membrane-associated V_1V_o complex but not with the soluble V_1 complex (Figure 8A, lanes 3 and 4). In addition, ATP caused partial dissociation of BiP from the complex; however, a nonhydrolyzable analog, ATP-γ-S, had no effect (Figure 8B). Although BiP frequently escapes into the soluble fraction during membrane isolation (Figure 2A, bottom), the absence of BiP association with the soluble V_1 complex indicates that (1) BiP does not bind to V_1 subunits and (2) BiP specifically binds to the membrane-bound V-ATPase, possibly with V_o subunits. The results suggest that BiP, a soluble chaperone, also participates in the folding or assembly of the membrane-bound V_1V_o-ATPase complex.

DISCUSSION

Calnexin Copurified with the V-ATPase

We have identified the protein that copurifies with the V-ATPase (Ward and Sze, 1992) as calnexin. Using MAbs against the protein, we found that (1) the 64-kD protein from roots (Figure 1) or a 67-kD protein from shoots (data not shown) is associated with the ER, as determined by its comigration with BiP on sucrose density gradients; (2) the 67-kD protein (from shoots) is an integral membrane protein that was solubilized by detergents (Figure 2A); and (3) assuming that ER vesicles are oriented right side out, trypsin digestion in the absence and presence of Triton X-100 indicated that a short section was exposed to the cytoplasm, whereas most of the molecule faced the lumen (Figure 2B). These biochemical features agree with the topology of mammalian calnexin as a type I membrane protein in which a large N-terminal region resides in the ER lumen and a short C tail protrudes into the cytoplasm (Bergeron et al., 1994). The identity of the integral protein was positively demonstrated by MAb 11A1 reactivity with a fusion protein containing the N-terminal half of Arabidopsis calnexin (Figure 3).

Isoforms of Calnexin in Oat

The variation in molecular masses of calnexin isolated from shoots (67 kD) and from roots (64 kD) suggests the presence of organ-specific isoforms in oats (Figure 5A). There is evidence of more than one form of calnexin within each organ because immunoblotting with MAb 11A1 consistently stained two polypeptides in roots and two or three polypeptides in shoots. Whether this reflects additional isoforms or post-translationally modified forms of one calnexin is unclear. Although the predicted molecular masses of plant and mammalian calnexins are similar at 60 to 65 kD, plant calnexins have an apparent molecular mass of 64 to 67 kD, as determined on SDS–polyacrylamide gels, whereas animal calnexins have an apparent size of 88 to 90 kD (Williams, 1995). The discrepancy could be caused by two stretches of 20 to 30 acidic residues in mammalian calnexin that are absent from the Arabidopsis protein at the N and C termini (Huang et al., 1993). These acidic residues could alter the amount of SDS binding to the protein (Williams, 1995), thus altering its mobility.

Calnexin and BiP Associate with V-ATPase Complexes

Molecular chaperones associate transiently with newly synthesized proteins that are folding or assembly intermediates and do not bind to fully folded, assembled proteins (Williams, 1995). The results presented here support our working hypothesis that calnexin acts as a chaperone in the folding and/or assembly of the V-ATPase complex. First, reciprocal immunoprecitation provided compelling evidence that calnexin associates with V-ATPase subunits. A MAb against calnexin precipitated the major subunits of the V-ATPase, whereas a MAb to subunit A alone precipitated the entire V-ATPase complex as well as calnexin (Figure 6) when membrane proteins were solubilized with Triton X-100. Because Triton X-100, but not SDS, retains the tertiary and quaternary structure of proteins, these results indicate that calnexin and the V-ATPase are parts of one large complex. Second, MAb 8B6 precipitated relatively more V-ATPase than did calnexin (Figure 6), indicating that only a fraction of the total V-ATPase was associated with calnexin at any given time. Although MAb 8B6 will indiscriminately precipitate all complexes containing subunit A, including fully assembled as well as partially assembled V-ATPases, chaperones should associate with newly synthesized proteins that are incompletely assembled (Williams, 1995). The result shown in Figure 6B is consistent with this idea, because calnexin is associated with a small population of the total V-ATPase in the cells. Third, calnexin appeared to associate with a V-ATPase precomplex that was slightly smaller in molecular mass than the fully active, mature V-ATPase. When membranes were Triton X-100 solubilized, calnexin associated with complexes ranging from 200 to 440 kD, as determined by estimates from sedimentation centrifugation (Figure 4B) and gel filtration. During purification by Sephacryl S-400, a 64-kD polypeptide, now recognized as calnexin, eluted immediately after the peak of activity (Ward and Sze, 1992), with an average size of 300 kD (J.M. Ward, data not shown). The largest calnexin–protein complex that we detected was ~500 kD (Figure 4B, lane 7). These results suggest that calnexin is not associated with fully assembled V-ATPases that are active. Rather, calnexin probably interacts with various forms of partially and nearly assembled V-ATPase complexes.

We do not know which V-ATPase subunit interacts with calnexin, although the most likely candidates are the 16-kD subunit, the integral 100-kD polypeptide, and other V_o subunits. Because calnexin prefers interacting with glycosylated proteins that carry Asn-linked oligosaccharides (Hammond et al., 1994; Williams, 1995; Helenius et al., 1997), it could associate with a 100-kD subunit, which is glycosylated in the bovine coated vesicle H^+-ATPase (Adachi et al., 1990). Furthermore, the 95-kD Vph1p in yeast has three potential N-linked glycosylation sites (Manolson et al., 1992). However, calnexin can also recognize polypeptide portions of incompletely folded proteins and transmembrane domains of the P glycoprotein when glycosylation sites are either absent or removed (Loo and Clarke, 1994, 1995).

Coimmunoprecipitation of BiP and V-ATPase by MAb 8B6 suggests that the translocation, folding, and assembly of the V-ATPase complex also depend on an ER lumen chaperone. Like other BiP-nascent protein interactions (Boston et al., 1996), the association of BiP with V-ATPase is sensitive to ATP (Figure 8B). BiP was coprecipitated only if V-ATPase was attached to the membrane but not associated with soluble V_1-ATPase, suggesting that BiP interacted with the V_o portion. Although BiP is required for the translocation of secretory proteins in the ER (Nguyen et al., 1991), it also acts as a molecular chaperone of integral membrane proteins, which include the P glycoprotein (Loo and Clarke, 1994) and the VSV G protein (Hammond and Helenius, 1994). The participation of additional ER lumen chaperones, such as calreticulin (Denecke et al., 1995) and GRP94 (Melnick et al., 1994; Boston et al., 1996), needs to be examined. Binding of several ER chaperones to the V-ATPase suggests that calnexin and BiP may recognize different structural elements exposed perhaps sequentially, as occurs in the VSV G protein during the progression of folding and assembly (Hammond and Helenius, 1994).

Evidence from yeast mutants defective in V-ATPase assembly indicates that other ER-associated proteins are required for the assembly of the V_o sector and perhaps the entire complex at the ER (Hirata et al., 1993; Hill and Stevens, 1994, 1995). In *vma21*, *vma22*, or *vma12* mutants, V_1 subunits accumulate in the cytosol, and the V_o 100-kD subunit is rapidly degraded by nonvacuolar proteases. Vma21p, Vma22p, and Vma12p could be chaperones specifically required for proper assembly of the V_o subunits in ER membranes, because the assembly and targeting of other integral membrane proteins are unchanged in the three mutants. Interestingly, the assembly of V-ATPase in baker's yeast may not depend on calnexin, because CNE1, which encodes a calnexin homolog, can be disrupted without affecting growth (Parlati et al., 1995). In contrast, *CNX1*, encoding calnexin in fission yeast, is essential for growth (Jannatipour and Rokeach, 1995). Whether homologs of Vma21p, Vma22p, and Vma12p exist in plants is unclear; however, proteins of 64 and 66 kD that coimmunoprecipitated with the V-ATPase using MAb 8B6 could be additional components of the assembly machinery (Figure 6A, lane 2, arrows).

A Working Model of V_1V_o Assembly

Our results provide additional information for a general working model of the synthesis and assembly of the V-ATPase. Several studies with yeast mutants support the separate sector assembly pathway in which the V_1 and the V_o sectors assemble separately before the formation of the V_1V_o-ATPase complex (Kane and Stevens, 1992). However, it is unclear whether (1) a fully assembled V_1 is attached to the V_o sector or (2) V_1 subunits are added to the V_o sector as partial V_1 complexes to build a complete V_1 sector. Because our

initial results do not allow us to differentiate between the two models, both possibilities are considered in our working model (Figure 9).

V_o is synthesized and assembled in the ER. The translocation, folding, and assembly of V_o occurs independently and may depend on folding and assembly machinery that includes calnexin, BiP, and additional unidentified partners. Calnexin and BiP most likely associate with the V_o portion that is exposed to the ER lumen, although calnexin may interact with the transmembrane domain of V_o subunits as well. Antibodies that immunoprecipitate V_o subunits, although not available yet, are needed to test whether calnexin initially associates with the V_o complex alone. Both chaperones participate, perhaps indirectly, in the assembly of V_1 subunits with V_o, because both BiP and calnexin are bound to the V-ATPase even after V_1 subunits are attached (Figures 6 and 8). This is demonstrated by the coprecipitation of both chaperones by an antibody against subunit A; however, it is unclear whether BiP and calnexin act sequentially or at the same time. The sequence and mode of V_1 assembly as well as the potential participation of cytoplasmic chaperones have yet to be examined. If calnexin and BiP are resident in the ER of oat seedlings, then our results indicate that V_1 subunits are assembled with the V_o sector at the ER. The possibility that other V-ATPases are assembled elsewhere could be considered if calnexin resides on other membranes of the secretory system in plants (Delmer et al., 1993). Calnexin dissociates from the V_1V_o complex after the V-ATPase is fully assembled and functionally active. The complete V-ATPase then transits from the ER to its final destination, such as the Golgi apparatus, various endomembrane compartments, and the vacuole, depending on targeting or retention machineries that have yet to be identified.

The association of calnexin and BiP with V-ATPase subunits suggests that they act as molecular chaperones in the translocation, folding, and assembly of the V-ATPase. Coprecipitation of both V_1 and V_o subunits with calnexin indicates that V_1 subunits, either singly or as a complex, are attached to the membrane integral V_o sector. Thus, the V_1V_o-ATPase is fully assembled at the ER and perhaps at the Golgi apparatus. The association of calnexin with the 100-kD plasma membrane H^+-ATPase suggests that calnexin may act as a chaperone for the insertion and folding of several transport proteins. Importantly, calnexin and BiP may serve as molecular chaperones for the folding and assembly of V-ATPases at the ER not only in plants but perhaps in eukaryotes in general.

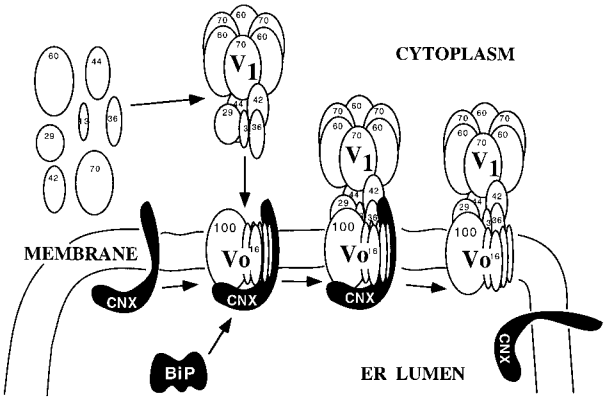

Figure 9. A Working Model for the Assembly of the V_1V_o-ATPase at the ER.

The integral ER protein calnexin (CNX) associates with integral membrane subunits (V_o) of domains exposed to the lumen or to transmembrane domains or both, whereas BiP binds to integral subunits exposed to the lumen. Calnexin, BiP, and other as yet unidentified chaperones may bind sequentially to facilitate the folding and assembly of the V_o subcomplex and the assembly of peripheral V_1 subunits or V_1 subcomplexes with the V_o. The chaperones then dissociate from the fully assembled V-ATPase. Numbers refer to molecular masses of V-ATPase subunits.

METHODS

Plant Material

Oat (*Avena sativa* var Ogle) seeds were germinated in the dark over an aerated solution of 0.5 mM $CaSO_4$ at 22°C. Shoots or roots were harvested after 3 or 4 days of growth.

Antibodies

Monoclonal antibodies (MAbs) had been generated to a V-ATPase purified by gel filtration from oat roots (Ward and Sze, 1992). MAbs 8B6 (or 7A5) and 2E7 recognized subunits A and B, respectively (Ward et al., 1992). MAbs 11A1 and 12A2 against an ~65-kD polypeptide were obtained at the same time (Ward, 1991). Polyclonal antibodies against the V-ATPase had been produced from a purified holoenzyme from oat roots (Randall and Sze, 1989). Polyclonal antibodies raised against *Arabidopsis thaliana* calnexin, maize plasma membrane H^+-ATPase, and tomato BiP were gifts from N. Hoffman (Carnegie Institute of Washington, Stanford, CA), R.T. Leonard (University of Arizona, Tucson), and M. Chrispeels (University of California, San Diego), respectively.

Isolation and Fractionation of Subcellular Membranes from Oat

Oat shoots or roots (50 gm fresh weight) were homogenized in a cold room at 4°C with a mortar and pestle in 100 mL of a medium containing 50 mM Hepes–bis–Tris propane (Hepes–BTP), pH 7.4, 250 mM sorbitol, 0.5% BSA, 0.1 mM phenylmethylsulfonyl flouride (PMSF), and 0.05 mM *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). The homogenate was strained through four layers of cheesecloth and centrifuged at 13,000*g* for 15 min to remove debris and mitochondria. The postmitochondrial supernatant was centrifuged at 80,000*g* for 45 min to pellet microsomal membranes. The pellet was washed twice and then suspended with 20 to 40 mL of a resuspension buffer

containing 25 mM Hepes–BTP, pH 7.4, 250 mM sorbitol, 0.1 mM PMSF, and 0.05 mM TPCK.

To separate membranes, 3 to 4 mL of a postmitochondrial supernatant (from 0.5 gm fresh weight tissue) was layered onto a 15 to 45% continuous sucrose gradient (12 mL) over a 1-mL 45% sucrose cushion. After centrifugation at 90,000*g* for 16 hr using a rotor (25,000 rpm; model SW28; Beckman Instruments, Palo Alto, CA), 0.7-mL fractions were collected and diluted with resuspension buffer to <10% of sucrose. Protein in each fraction was directly precipitated with 20% trichloroacetic acid (TCA; final concentration) and washed with cold acetone. Protein concentration was determined using the Bio-Rad protein assay solution.

Treatment with Detergent, Salt, or Trypsin

Microsomal membranes (0.5 mg protein in 0.5 mL) were incubated with a final concentration of 0.2 or 1% Triton X-100, 0.1% SDS, 0.5 M NaCl, 2.5 M urea, or resuspension buffer (mock treatment as a control) for 30 min at 4°C. Treated samples were centrifuged at 100,000*g* for 40 min using a rotor (55,000 rpm; model TL100.3; Beckman Instruments). The supernatant and pellets were collected, and the proteins were precipitated with 20% TCA and washed with cold acetone. Proteins were resuspended with 1 × SDS gel sample buffer, and one-tenth of each sample was analyzed by SDS-PAGE and immunostaining.

For trypsin digestion, microsomal vesicles (0.75 mg of protein) were incubated with trypsin (100 μg/mL; type XIII; Sigma) in a 0.5-mL reaction volume for various periods of time in the presence (control) or absence of 2% Triton X-100. After 0, 2, 5, 10, 15, and 30 min at room temperature, 50 μL of treated vesicles was removed and immediately mixed with an equal volume of 2 × SDS gel sample buffer, boiled for 5 min, and then stored at 80°C. Approximately 15 to 25 μg of protein from each treated sample was used for SDS-PAGE and immunoblotting.

Fractionation of Triton X-100–Solubilized Proteins

Usually 2 mL of membranes containing 2 to 4 mg protein was solubilized with 0.5 mL of 20% Triton X-100 (final concentration of 4%) in 25 mM Hepes–BTP, pH 7.4, and 250 mM sorbitol for 30 min at 4°C. Unsolubilized material was removed by centrifugation at 150,000*g* for 45 min using a rotor (45,000 rpm; model TY65; Beckman Instruments). The Triton X-100–solubilized proteins (0.4 mL) were loaded onto a 12-mL linear 5 to 26% sucrose gradient containing 1% Triton X-100. Sometimes microsomal membranes were solubilized with 1% SDS and 2% Triton X-100. The SDS-solubilized proteins were loaded onto the same sucrose gradient containing 1% Triton X-100 plus 0.1% SDS. Sucrose solutions were prepared with resuspension buffer containing 25 mM Hepes–BTP, pH 7.2, 0.1 mM PMSF, and 0.05 mM TPCK. The gradients were centrifuged at 200,000*g* for 16 hr using a rotor (40,000 rpm; model SW40; Beckman Instruments). Fractions of 0.5 mL were collected, and proteins were precipitated by 20% TCA and washed with acetone. Pellets were resuspended in 1 × SDS gel sample buffer, and one-half of each fraction was analyzed by SDS-PAGE and immunostaining.

Immunoprecipitation

All of the procedures were conducted at 4°C. Membranes were isolated from shoots because more protein per gram fresh weight can be recovered from shoots than roots. A freshly prepared microsomal pellet was suspended in a solubilization mixture containing 25 mM Hepes–BTP, pH 7.4, 250 mM sorbitol, 0.2% BSA, 0.1 mM PMSF, 0.05 mM TPCK, leupeptin (1 μg/mL), pepstatin (5 μg/mL), aprotinin (1 μg/mL), and chymostatin (5 μg/mL). Usually, 8 mL of membranes (8 to 12 mg of proteins) was solubilized with 2 mL of 20% Triton X-100 (final concentration of 4%) in 25 mM Hepes–BTP, pH 7.4, and 250 mM sorbitol for 30 min. Unsolubilized material was removed by centrifugation at 150,000*g* for 45 min (45,000 rpm). The supernatant was collected and used immediately for immunoprecipitation.

Immunoprecipitation was performed according to the method of Feng et al. (1995) with some modifications. Solubilized proteins (0.8 to 1 mL) were precleared by incubating with 30 μL of protein A–Sepharose bound to rabbit anti–mouse IgG (PAM) for 1 hr followed by pelleting to remove proteins that bound nonspecifically to protein A and IgG. PAM was prepared as follows. Protein A–Sepharose (0.75 gm) (P3391; Sigma) was suspended in 10 volumes of PBS. Protein A (0.8 mL) was then incubated for 1 hr with 100 μL of AffiniPure rabbit anti–mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 4°C. The mixture was washed three times with PBS containing 0.1% Triton-X 100 by pelleting in a microcentrifuge for 30 sec. After the final wash, the protein A that bound to rabbit anti–mouse IgG (or PAM) was resuspended with 0.4 mL of PBS containing 0.02% sodium azide. The precleared supernatant was then incubated with each antibody (usually 100 μL) for 1 hr on a shaker at 4°C. Immunoprecipitation was initiated by incubation with 30 μL of PAM for another hour. The mixture was microcentrifuged for 30 sec, and the supernatant was completely removed by aspiration. Pellets were washed three times with Hepes buffer containing 25 mM Hepes–BTP, pH 7.4, 250 mM sorbitol, 0.1 mM PMSF, 0.05 mM TPCK, and 0.1% Triton X-100. The pellets were resuspended in 40 or 50 μL of 1 × SDS gel sample buffer. After boiling for 5 min, the beads were pelleted, and the supernatants (one-half or entire volume) were subjected to 11% SDS-PAGE and immunostained.

SDS-PAGE, Silver Staining, and Immunoblotting

SDS-PAGE and immunoblotting were conducted as described previously (Herman et al., 1994), except the SDS sample buffer contained 62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.002% (w/v) bromophenol blue. Proteins were stained either with Coomassie Brilliant Blue G 250 or with silver (Oakley et al., 1980). Unstained proteins gels were blotted onto Immobilon-P membrane (Millipore, Bedford, MA), and the blots were incubated with specific antibodies, as described in the figure legends. The blots were then probed with either goat anti–mouse IgG or goat anti–rabbit IgG conjugated to alkaline phosphatase, and color was developed with 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium (Herman et al., 1994).

Expression of cDNA Clones Encoding Arabidopsis Calnexin in Bacteria

cDNA clones encoding the calnexin homolog of Arabidopsis (Huang et al., 1993) were provided by N. Hoffman. A 1161-bp BglII fragment (base pairs 42 to 1203), which included 45 bp of the 5′ untranslated region plus two-thirds of the open reading frame, was subcloned in frame into a histidine-tagged fusion vector pET28 (Novagen, Madison, WI) (see Figure 1A). A 615-bp SalI-HindII fragment (base pairs 918 to 1533), corresponding to an internal region of calnexin, had

been subcloned into the expression vector pMAL-cRI by Huang et al. (1993) to create a fusion with the maltose binding protein (Figure 1A). *Escherichia coli* BL21 (DE3) and TB-1 were transformed with pET28 and pMAL-cRI vector constructs, respectively, and incubated with or without isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C to induce expression of the fusion proteins. After various intervals, 1 mL of bacteria was collected and stored at −80°C. Cells were pelleted and lysed with 0.1 mL of 1 × SDS sample buffer. The supernatants (~20 μg each) of the lysates were analyzed by SDS-PAGE and immunostaining.

ACKNOWLEDGMENTS

We thank John M. Ward (see Ward, 1991) for identifying MAbs against a 65-kD protein (now known as calnexin) and Neil Hoffman for providing cDNA clones and antibodies of Arabidopsis calnexin. Polyclonal antibodies raised against plasma membrane H^+-ATPase and BiP were generous gifts from Robert T. Leonard and Maarten Chrispeels, respectively. This work was supported in part by U.S. Department of Agriculture National Research Initiative Grants No. 94-37304-1071 and No. 97-35304-4523 to H.S. and by the Maryland Agricultural Experiment Station.

Received July 14, 1997; accepted October 31, 1997.

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