Primary Structure and Characterization of an *Arabidopsis thaliana* Calnexin-like Protein*

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A cDNA clone (p2E-83) encoding a protein (CNX1p) related to the microsomal Ca\(^{2+}\)-binding protein, calnexin, was isolated from an *Arabidopsis thaliana* expression library. Southern and Northern hybridization indicated that CNX1 is a single-copy gene encoding a message of 1900 nucleotides. The open reading frame encodes a polypeptide with 530 amino acids, a molecular mass of 60.5 kDa, and overall 48% identity to dog calnexin. Both animal calnexin and CNX1p contain a large luminal domain followed by a single potential membrane-spanning domain near the C terminus and a small C-terminal domain exposed to the cytoplasm. The *in vitro* translation product from the cloned cDNA yielded a polypeptide of 67 kDa that was co-translationally imported into dog microsomes and processed to a 64-kDa product. Antibodies generated against the C-terminal half of the protein cross-react with an identically sized protein present in the microsomal fraction from *Arabidopsis*. Both the imported and native proteins are cleaved by trypsin to a 59-kDa product indicating that the gene product was indeed correctly processed and translocated into dog microsomes and that the membrane topology of CNX1p resembles that of dog calnexin. The presence of a calnexin-like protein within the plant kingdom indicates that this protein is widespread and involved in processes fundamental to all eukaryotes.

Calnexin is one of the major integral membrane proteins localized in the endoplasmic reticulum (1). It has a single transmembrane domain near the C terminus, a long N-terminal domain within the ER lumen, and a short C-terminal domain in the cytosol. The cytosolically exposed domain can be serine-phosphorylated *in vitro*. Calnexin binds Ca\(^{2+}\) and is similar in sequence to calreticulin (2), a major Ca\(^{2+}\)-binding protein in the lumen of the ER.

Possible functions for calnexin have been suggested by a number of recent studies. Wada et al. (1) coimmunoprecipitated and copurified calnexin in a complex with three other transmembrane polypeptides. Two of the polypeptides, SSR\(\alpha\) and SSR\(\delta\), had been previously identified (3) and were proposed to be involved in translocating nascent polypeptides into the ER; the third protein had not been previously characterized. SSR\(\alpha\) was found to also bind Ca\(^{2+}\) ion and be phosphorylated in the cytosolic C-terminal region only (1). As a result, Wada et al. (1) suggested that the complex may act as a signal transducer sensing or affecting changes in intraluminal calcium levels. Based on the observations that depletion of luminal Ca\(^{2+}\) led to the secretion of soluble resident ER proteins (4–6), Wada et al. (1) also suggested that calnexin may play a role in the Ca\(^{2+}\)-dependent retention of these proteins.

Other studies have indicated that calnexin functions as a molecular chaperone. Degen and Williams (7) described an 88-kDa ER protein (p88) that participates in the assembly of murine class I histocompatibility molecules. They suggested that p88 may either promote proper assembly of class I molecules or retain them within the ER until assembly of the ternary complex of heavy chain, \(\beta\)-microglobulin, and peptide ligand is complete (8). It was recently revealed that p88 and calnexin resemble the same protein (9–11). Hochstenbach et al. (10) also showed that calnexin was transiently associated with a number of partially assembled membrane complexes. Hochstenbach et al. (10) and Ahluwalia et al. (9) have noted that calnexin may function analogously to the immunoglobulin-binding protein, BiP, as a general molecular chaperone. BiP is a soluble luminal protein that facilitates folding and assembly of proteins during their transit through the ER and retains proteins within the ER, in a Ca\(^{2+}\)-dependent manner, until folding is complete (6, 12).

The current knowledge about calnexin is quite limited with regard to its actual cellular function, its structural features, and its regulation. It remains to be demonstrated whether calnexin occurs ubiquitously in eukaryotic organisms. If so, the common features may provide insight into its molecular structure and function.

In the present study, we report the primary structure, deduced from cDNA sequence, of the calnexin-like protein, CNX1p, in the higher plant *Arabidopsis thaliana*. The plant protein exhibits high sequence identity to mammalian calnexin. Immunoblot analyses and *in vitro* import studies indicate that CNX1p is localized in the microsomal fraction. The *in vitro* import studies also confirm its predicted membrane topology and indicate that the plant calnexin contains a functional signal sequence.

**EXPERIMENTAL PROCEDURES**

**Materials**—The *A. thaliana* (L. cv. Columbia) cDNA library, constructed in a YES (13), was a gift of Dr. John Mulligan, Department...
of Biochemistry, Stanford University. Sequence (version 2.0 T7 DNA polymerase) for sequencing and goat-anti-rabbit IgG-alkaline phosphatase were from U. S. Biochemical Corp. α-32P-dATP, [32P] methionine, and [α-32P]dCTP were from Amersham Corp. The expression vector pMAL-cRI and amylose resin were from New England Biolabs. TrpX and α-mannosidase from Boehringer Mannheim. Poly(A)-poly(U) was from Pharmacia.

Preparation of Antibodies—Total chloroplast envelope membranes were prepared from spinach leaves essentially according to the method of Keegstra and Youniss (14) with the following modifications. Crude envelope membranes were pelleted by centrifugation at 75,000 rpm for 3 min in a TL100.3 rotor. Membranes were resuspended in 0.2 M sucrose containing 20 mM Tris, pH 8.0, and 0.2 mM EDTA (TE) and layered on top of 1.1 M sucrose + TE. Samples were centrifuged as above, and the yellow membranes at the interface were collected, diluted in TE, and concentrated by centrifugation as above. The envelope polypeptides were size-fractionated by SDS-PAGE (13), and proteins of 55-75 kDa were electroeluted. Antisera against the 55-75-kDa proteins were raised in a single rabbit (16). The IgG fraction was purified from serum by ammonium sulfate precipitation and DEAE-Sepharose chromatography (17).

Antibodies were also generated against a peptide expressed from a portion of the calnexin-like cDNA, pTE6-83. The 615-bp SalI-HindIII fragment (bp 918-1533) from pTE6-83 (see Fig. 1B) was subcloned into the expression vector, pMAL-cRI, creating a fusion with maltose-binding protein. After verification by DNA sequencing, the fusion protein was overexpressed in Escherichia coli strain TB-1 and purified through an amylose column according to the manufacturer’s directions. The fusion protein (1 mg/ml) was cleaved by incubation with factor Xa (0.01 mg/ml) at room temperature for 3 h in a buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, and 2 mM CaCl2. Maltose-binding protein was separated from antigen by a second passing over the amylose column, and the antigen in the effluent was further purified by SDS-PAGE followed by electro-elution. The eluted protein was used to generate polyclonal antibodies, and IgG was prepared as described above. Antigen was immobilized on cyanogen bromide-activated Sepharose beads (17) and used to immunoaffinity purify the IgG fraction. Antibodies were eluted from the column using 0.1 M glycine solution, pH 2.5 (17).

Screening of cDNA Library and Sequencing of cDNA Clones— Rabbit IgG produced against the 55-75-kDa envelope polypeptides were used to screen the Arabidopsis cDNA λ YES library expressed in E. coli strain JM107 bearing the cDNA, pTE6-83 (see Fig. 1B) with the following modifications. 1) Filters were successively washed for 6 h in Tris-buffered saline (TBS; 10 mM Tris (pH 7.5), 150 mM NaCl), blocked for 30 min in TBS + 2% nonfat dry milk, and rinsed in TBS prior to incubation with the primary antibody; 2) 0.2% Nonidet P-40 was used as a blocking agent in subsequent steps instead of 2% nonfat dry milk; 3) antibodies were used at a dilution of 1:250. From approximately 500,000 plaques, 18 positive clones were isolated for further characterization. Plasmids were liberated from plaque DNA after infection of KCM/MI07 with the plaque isolate, and cDNA insert size was characterized by EcoRI and XhoI digests. To type the phage clones by DNA hybridization, the E. coli host strain was plated onto LB-agar, and 1 μl of individual plaque isolates were spotted in a grid pattern. Plates were incubated overnight at 37 °C, plaques were lifted onto nitrocellulose filters, and filters were hybridized against DNA probes made from the individual clones. These clones were grouped into three classes of non-cross-hybridizing clones. The type reported here was isolated seven independent times. Both strands of the longest clone, pTE6-83, were sequenced (19).

Immunoblotting of Plant Subcellular Fractions—Plasm microsomal fractions were prepared according to Gallagher et al. (20) from 10- to 15-g tissue grinding or according to a modified procedure. Chlroplasts were isolated according to Cline (21) and fractionated into membrane and stroma essentially as described previously (22). Samples were electrophoresed and immunoblotted as described (18) with slight modifications. Briefly, the nitrocellulose was blocked with 2% bovine serum albumin in TBS for 20 min at room temperature, followed by incubation with the immunoadfinity-purified antibodies (from above) at 2 μg of protein/ml in TBS containing 2% bovine serum albumin, 0.3% Nonidet P-40, 0.1% SDS, and 0.05% sodium azide for 12 h at 4 °C. After three 5-min washes (1 × TBS, 1 × TBS + 0.2% Nonidet P-40 + 0.1% SDS, and 1 × TBS), the nitrocellulose was incubated with goat anti-rabbit IgG coupled with alkaline phosphatase (1:10,000 dilution) in 1 × TBS containing 2% bovine serum albumin, 0.2% Nonidet P-40, and 0.05% sodium azide for 1 h at room temperature. It was then washed and developed as described (18).

In Vivo Translation and Import—The insert from pTE6-83 was subcloned into the EcoRI and XhoI sites of pSP73, transcribed in vitro using SP6 RNA polymerase (23), and translated in vitro in rabbit reticulocyte lysate in the presence of [35S]methionine with or without dog pancreatic microsomal membranes following procedures suggested from Fromega. Peptatin, 10 μM, was added during translation to reduce proteolysis of the precursor. After 60 min, 5% of the reaction (1.25 μl) was incubated in 15 μl (final volume) of 25 mM Hepes pH 8.0 containing 250 mM sucrose for 30 min at 0 °C. Where indicated, trypsin and Triton X-100 were added to 0.1 mg/ml and 0.2%, respectively. Reactions were terminated by the addition of SDS solubilization buffer followed by immediate boiling for 5 min. Samples were run on SDS-PAGE, and gels were dried and autoradiographed.

Northern Blot Analysis—Total RNA was isolated according to Verwoerd et al. (24) from green shoots of A. thaliana (L. cv. Columbia) grown under continuous light. Total RNA was separated electrophoretically (25 μg/lane) in a 1.2% (w/v) agarose gel containing 5% (v/v) formaldehyde, blotted onto Hybond-N membrane by capillary transfer, and hybridized to full-length pTE6-83 insert (2 × 106 cpm/ml in solution containing 0.75 mM NaCl, 0.075 mM sodium citrate (5 × SSC), 0.5% SDS, 5 × Denhardt’s solution (25), and 50 μg/ml salmon salmon sperm DNA) at 65 °C for 16 h in solution containing 0.1 × SSC, 0.1% SDS at 63 °C for 30 min.

Genomic DNA Analysis—Genomic DNA was isolated according to Saghai-Marof et al. (26) from whole plants of A. thaliana (L. cv. Columbia) grown aseptically in liquid culture in Murashige and Skog salts supplemented with 0.2% sucrose under continuous light, at 18 °C, with shaking. DNA was digested with EcoRI or BglII and 0.1 μg/lane was separated on a 0.75% (w/v) agarose gel, transferred onto Hybond-N membrane, and hybridized to 32P-labeled full-length pTE6-83 insert (106 cpm/ml in 6 × SSC, 0.5% SDS, 5 × Denhardt’s solution) (25), and 100 μg/ml salmon sperm DNA) for 15 h at 68 °C. The final wash was in 0.1 × SSC containing 0.1% SDS at 68 °C.

RESULTS

Sequence and Structural Analyses—We have been using an immunological approach to isolate clones encoding chloroplast envelope polypeptides. While screening an Arabidopsis cDNA expression library with polyclonal antibodies made against envelope polypeptides in the apparent molecular mass range of 55-75 kDa, we isolated seven types of non-cross-hybridizing clones. One of these types encodes a putative ATPase from the chloroplast inner envelope and will be described elsewhere. A second type was isolated seven independent times and is the subject of the present report. The longest of the clones of the second type, designated pTE6-83, was further characterized by sequencing. Fig. 1A shows the nucleotide and deduced amino acid sequences from the 1850-bp cDNA clone. Although the open reading frame extends to the 5’ end of the cDNA, we believe the first ATG, beginning with nucleotide 87, is the initiating Met for reasons that will be described below. The cDNA would encode a protein of 530 amino acids with a calculated molecular mass of 60,481 Da.

Searching the GenBank data base revealed that the protein predicted by pTE6-83 shares significant sequence identity with that of dog calnexin (1), and, hence, we designated the calnexin-like gene and protein, CNX1 and CNX1p, respectively. CNX1p is 47.6% identical to dog calnexin (for alignment, see Fig. 2). Like dog calnexin, CNX1p also resembles calreticulin; it shares 38.7% identity with rabbit calreticulin (2).

Dog calnexin begins with a 20-residue signal sequence (1). The deduced sequence of CNX1p also begins with what appears to be a classical signal sequence. The first Met is followed in succession by positively charged and hydrophobic residues (27). As described below, CNX1p contains a functional signal sequence that is processed by dog microsomes.
Processing sites of signal sequences frequently occur after a small amino acid, and signal sequences are generally longer than 15 residues (27). Based on this information, a probable processing site would be after Ser1.

For the most part, the middle of the deduced calnexin proteins are highly conserved, while the first 100 and the last 150 residues are highly diverged. There are seven domains that have been conserved between the two proteins ranging from 50 to 75% identity (labeled A-G in Fig. 2). Domain D contains the most extensive region of amino acid identity. This domain is also highly conserved between calnexin and calreticulin, which has been called the P domain due to the frequency of proline residues (28). Wada et al. (1) noted that in this domain, calnexin and calreticulin share three internally repeated sequences of KPEDWD and GXW. We note that both of these repeats are part of two larger motifs having the consensus sequences DP(E/D)(A/D)XPEDWD(D/E) and GXWXXPXDNP, respectively. In domain D of calnexin and CNXlp, the first motif is repeated three times followed by four repeats of the second motif (Fig. 2).

CNXlp (predicted pl = 4.62) is less acidic than dog calnexin (predicted pl = 4.27) and lacks the acidic domains found at the N and C termini (Fig. 2). An acidic C terminus is found in calreticulin as well as a number of other Ca²⁺-binding ER proteins (28). In contrast, the C terminus of CNXlp is basic. As was found for mammalian calnexin, the hydropathy plot of CNXlp (Fig. 1C) predicts a single transmembrane domain near the C terminus. Although the C termini are generally divergent, there are short regions of sequence identity just flanking the transmembrane domain (amino acids 456-465 and domain F) and at the very C terminus (domain G). The significance of domain F is unclear, but domain G may include the basic residues in domain G that contribute to retaining transmembrane proteins in the ER.

**Southern Blot Analysis—Genomic DNA isolated from whole plants of Arabidopsis was digested with EcoRI or BglII and subjected to Southern blot analysis using the full-length cDNA (panel B), only a single 7.9-kb band was cut internal to the cDNA, and hydrophilicity plot of CNXlp (panel C).**
FIG. 3. Southern (panel A) and Northern (panel B) blot analyses of nucleic acids from Arabidopsis. In panel A, Arabidopsis DNA (5 μg) was digested with EcoRI or BglII, separated on an agarose gel, and transferred onto a nylon membrane as described under "Experimental Procedures" using the entire insert from pTE6-83 as a probe. The size of the bands was estimated using λ DNA digested with BstEII as a standard. In panel B, total RNA (25 μg) was separated in a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized to the pTE6-83 insert as described under "Experimental Procedures." The transcript size was estimated using RNA standards from Bethesda Research Laboratories.
not recognize the antigen, though it did recognize some low molecular weight proteins in both plants and an 80-kDa polypeptide in the Arabidopsis microsomal fraction. These bands are stronger in the preimmune blot, presumably because the preimmune serum was not affinity-purified. The antiserum recognized the 26-kDa antigen (lane 1) and strongly cross-reacted with a 64-kDa protein in the Arabidopsis microsomal fraction (lane 2) and a 69-kDa protein in the pea microsomal fraction (lane 3). Neither of these bands was detected by the preimmune serum. Antiserum did not cross-react with any proteins in the membrane or soluble chloroplast fractions. This provides strong evidence that CNX1p is a microsomal protein. Furthermore, pea microsomes contain an antigenically related protein that has a different electrophoretic mobility than CNX1p.

In Vitro Translation and Import Studies of CNX1 Gene Product—To establish if CNX1p contains a functional signal sequence, we examined whether the in vitro translated protein would be imported into dog microsomes. For comparison, we also examined whether the protein would be imported into pea chloroplasts. The cDNA encoding CNX1p was transcribed in vitro and translated in the presence of microsomes or chloroplasts to test for co-translational import. The RNA was also translated in the absence of membranes to test for posttranslational import into the two organelles. Uptake was established by treating the reaction mixture with trypsin and analyzing samples on SDS-PAGE.

Translation of the mRNA from pTE6-83 in the absence of the microsomes yielded a 67-kDa radiolabeled protein as the predominant product (Fig. 5, lane 1). Rabbit reticulocyte lysate appears to have some processing activity since some of the translation product is cleaved to a 64-kDa peptide, the same size as the mature protein. The processing could be partly inhibited by inclusion of 10 μM pepstatin during translation (data not shown). When the translation was carried out in the presence of microsomes, most of the 67-kDa protein was processed to a 64-kDa protein (Fig. 5, lane 3). Trypsin treatment completely digested both the 67-kDa precursor and 64-kDa peptide when microsomes were not present (Fig. 5, lane 2) but degraded the 64-kDa protein to a 59-kDa peptide in the presence of microsomes (Fig. 5, lane 4). When detergent was included to solubilize the microsomes, the 64-kDa band was completely digested (Fig. 5, lane 5). These results indicated that CNX1p can be imported into and processed by dog microsomes and has type I topology (27). We found that CNX1p was neither imported into microsomes or chloroplasts posttranslationally nor was it imported into chloroplasts cotranslationally (data not shown). These results provide further evidence that CNX1p is a microsomal protein and not a chloroplast protein despite how it was originally isolated.

To compare the topology of the native protein with that of radiolabeled CNX1p imported into microsomes, dog microsomes containing imported CNX1p were mixed with Arabidopsis microsomes, and the samples were treated with or without trypsin and analyzed by both immunoblot and autoradiography (Fig. 6). The arrows in Fig. 6 point to the bands that are specifically detected by the immune serum. These bands co-electrophoresed with the radioactive bands before and after trypsin treatment. This strongly suggests that the dog microsomes are processing CNX1p at the same site and integrating the protein into the membrane with the same topology as the native Arabidopsis protein. These data also indicate that pTE6-83 encodes a protein that is full-length.

Expression of Plant Calnexin Protein in Different Tissues—Microsomal fractions were isolated from green leaves, green stems, and etiolated leaves of pea plants and subjected to immunoblot analysis using the purified antibodies against CNX1p. The pea calnexin-like protein was present in all three samples at approximately the same level (Fig. 7).

DISCUSSION

While screening an A. thaliana expression library for cDNA clones that encode chloroplast envelope polypeptides, we re-
pestedly isolated a cDNA clone with an open reading frame encoding a calnexin-like protein. However, in mammalian cells, calnexin is localized in the ER. Using antibodies generated against the C-terminus of CNX1p, we observed that a single peptide from plant microsomes specifically cross-reacted with the immune serum, but we were unable to detect any antigenically related polypeptides in the membrane or soluble fraction from chloroplasts. We further demonstrated that CNX1p contains a functional signal sequence but not a functional chloroplast transit peptide. Together these data indicate that CNX1p is localized in the microsomes and not the chloroplast. It is possible that the chloroplasts used for the preparation of an igen were contaminated with ER, and because CNX1p is very abundant and possibly very antigenic, the corresponding cDNA was isolated at a high frequency.

Although we did not explicitly determine whether pTE6-83 represents the entire cDNA, the following observations support the conclusion that the cDNA encodes the entire protein and 86 nucleotides of the 5'-untranslated region. First, from Northern analysis, we observed a single hybridizing species that was approximately the same size as the cDNA. Second, the transcript encodes a protein with a functional and cleav-

able signal sequence, and, third, after import into microsomes, CNX1p co-electrophoreses with the native Arabidopsis protein.

Mammalian calnexin is clearly a Ca²⁺-binding protein, though it lacks the Ca²⁺-binding EF-hand motif of calmodulin (31). Likewise, no EF-hand motif is present in CNX1p. Calnexin and CNX1p show their highest amino acid identity in domain D. This region is also conserved between calnexin and calreticulin, where it has been shown to contain a low capacity high affinity Ca²⁺-binding domain (32). Therefore, CNX1p is also likely to be a Ca²⁺-binding protein. The precise site responsible for Ca²⁺ binding has yet to be determined in any of the aforementioned proteins. Calnexin, calreticulin, and CNX1p all contain at least two types of repeats in this domain, DP(E/D)(A/D)XKPEDWD(D/E) and GXWXXP-XIDNP, that may play a role in this function (28).

Like calnexin, CNX1p contains a single transmembrane domain near the C terminus. This is predicted from sequence analysis and supported by tryptic digestions of native protein in plant microsomes and protein imported into dog microsomes in vitro. Trypsin cleavage results in a 5-kDa reduction in the apparent molecular mass of the mature protein. Based on trypsin cleavage at Lys⁴⁹⁴, the first site after the predicted transmembrane domain, the calculated product would have a 4.6-kDa shift in molecular mass in good agreement with the observed result. Thus, it is likely that the cytosolically exposed C-terminal region is 42 amino acids in CNX1p, whereas this domain is 89 amino acids in dog calnexin.

Many Ca²⁺-binding proteins, including mammalian calnexin, calreticulin, calusequastrin, BiP, endoplasmic, and protein disulfide isomerase, have clusters of acidic residues at their C termini (28). In calreticulin, the C-terminal acidic domains have been shown to bind Ca²⁺ with low affinity but high capacity (32). Although calnexin has an acidic C terminus, this domain is localized in the cytosol where the Ca²⁺ levels are low and, hence, is unlikely to function as a low affinity binding site. The acidic domain at the N terminus of calnexin is in the lumen of the ER and conceivably could be involved in low affinity Ca²⁺ binding. CNX1p lacks both of the acidic domains found in calnexin and, hence, is unlikely to be involved in low affinity Ca²⁺ binding. Because plants store much of their Ca²⁺ in the vacuole and cell wall rather than the ER as animals do (33), plants may have a different demand for low affinity Ca²⁺-binding sites in the ER.

A number of investigators have observed a large discrepancy between the predicted molecular mass of Ca²⁺-binding proteins and the apparent molecular mass based on SDS-PAGE (1, 28). In the case of dog calnexin, the mature protein runs as a 90-kDa (hence called p90) band, 24 kDa greater than its sequence-predicted molecular mass of 65 kDa (1). It has been suggested that the discrepancy arises due to the abundance of acidic residues in the protein that presumably interfere with SDS binding, thereby reducing the protein mobility in SDS-PAGE (1). Consistent with this idea, CNX1p, which lacks two major acidic domains found in dog calnexin, runs as a 64-kDa mature protein, only 6.5 kDa greater than its sequence-predicted molecular mass.

Calnexin has so far been found in dog, mouse, and human (1, 9–11). We now report the existence of a calnexin-like protein in two plant species. The high sequence identity and conserved structural features between CNX1p and calnexin as well as localization of both the plant and animal polypeptides to microsomes make it likely that CNX1p is a plant homologue of calnexin. We expect that calnexin is probably ubiquitous among eukaryotic organisms and conserved through evolution. We found it similarly expressed in differ-
sufficiently diverged from its mammalian counterpart, prompting the speculation that calnexin functions as a general molecular chaperone. Consistent with this idea, we observe that calnexin is an abundant component of plant microsomes, suggesting that it is involved in processes common to both plants and animals. Mammalian calnexin proteins are conserved to a degree that it is not possible to identify conserved domains that contribute to calnexin function. The deduced plant sequence is sufficiently diverged from its mammalian counterparts that conserved sequences are now evident and can be further explored by structure-function studies.

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