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Purification, sequencing and functions of calreticulin from maize

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Abstract

The most abundant proteins in the lumen of the endoplasmic reticulum (ER) are thought to be molecular chaperones, some of which might also be involved in calcium storage and release. We have purified calreticulin from maize by ion exchange and reverse-phase chromatography. Identity with plant and animal calreticulins was confirmed by N-terminal amino acid sequencing and it was shown to bind calcium with a calcium overlay technique. An antiserum raised to the purified protein was used to screen an expression library and the full coding sequence for maize calreticulin was determined from the clones selected. The sequence shows 96% identity to barley calreticulin and 55% identity to animal calreticulins. The three major functional regions are conserved, as are targeting and retention features. When visualized by indirect immunofluorescence microscopy, calreticulin was found to be confined to the ER and nuclear envelope of maize root cells. It was distributed throughout the ER compartment and we found no evidence of calreticulin-enriched areas of ER, such as might be associated with specialized calcium storage domains. Increasing or decreasing extracellular calcium did not induce measurable changes in calreticulin levels. In addition, maize calreticulin, as well as other recognized chaperones, was shown to bind to denatured protein and could be eluted specifically by nucleoside trisphosphates.

Key words: Endoplasmic reticulum, calcium-binding protein, immunofluorescence, targeting, *Zea mays* L.

Introduction

A number of proteins are present ubiquitously in the lumen of the ER in eukaryotic cells. These include representatives of the heat-shock protein 90 (hsp 90) family such as the mammalian grp 94, luminal binding protein (BiP), protein disulphide isomerase (PDI), and calreticulin. Collectively, these luminal proteins have been termed reticuloplasmins (Koch, 1987; Macer and Koch, 1988) and each carries a C-terminal targeting signal to effect their retention within the ER, usually either HDEL or KDEL depending on the organism (Pelham, 1989). Plant homologues of each of these reticuloplasmins have been identified and some studied in detail. A luminal hsp90 has been purified from *Catharanthus roseus* (Schröder *et al.*, 1993). Sequences for a number of BiP isoforms and for PDI have been determined and their cell biology and roles as chaperones recorded (Shorrosh and Dixon, 1992; Shorrosh *et al.*, 1993; Boston *et al.*, 1991; Denecke *et al.*, 1991; Fontes *et al.*, 1991; Li *et al.*, 1993; Pedrazzini *et al.*, 1994). Calreticulin has been comparatively poorly characterized. There are two preliminary reports of identification in plants by using antisera raised against the mammalian protein (Allen and Tiwari, 1991; Zwialek *et al.*, 1993) and a report of purification and partial N-terminal sequence matches of two polypeptides from spinach (Menegazzi *et al.*, 1993). At the nucleic acid level, partial length cDNAs from random clone sequencing were identified as calreticulin (Park *et al.*, 1993) and incomplete, but much longer cDNAs from barley have been isolated and used to show that calreticulin is up-regulated during embryogenesis (Chen *et al.*, 1994). No evidence of function had been presented until very recently, when tobacco calreticulin was characterized

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(Denecke *et al.*, 1995). Again, the sequence data are incomplete, starting after the signal peptide. This is due in part to their protocol, choosing primers for polymerase chain reaction oligonucleotides from N-terminal sequence data. In their paper, evidence was shown of proteins associating with calreticulin and the authors suggested that it might have a role as a molecular chaperone in plants, just as evidence suggested for the protein in animal cells. Our data support and extend these observations.

The protein was first identified as a luminal calcium-binding protein in animal cells and, being relatively abundant, is thought to be a major site for calcium storage in the ER lumen of non-muscle cells. As a consequence, calreticulin has been used as a marker for subdomains of the ER specialized for calcium storage (Arber *et al.*, 1992). The animal protein has both a high affinity binding site for calcium and abundant low affinity binding sites (Michalak *et al.*, 1992). Calcium from this intracellular store can be mobilized rapidly in response to appropriate stimulation such as by stimuli initiating the inositol (1,4,5) trisphosphate signal cascade. A comprehensive review of mammalian calreticulin and its properties has been published by Michalak *et al.* (1992).

The plant ER also stores calcium (Bush *et al.*, 1989) and uptake and storage of calcium has been found to be hormonally regulated in barley aleurone ER (Bush *et al.*, 1989, 1993). The Ca^{2+}-ATPase necessary for calcium uptake into plant ER has been characterized (Evans, 1994; Logan and Venis, 1995), but little is known about calcium storage proteins or their distribution. Some evidence for specialized ER domains has been discussed (referred to as cortical ER (Hepler *et al.*, 1990) or calciosomes (Allen and Schumm, 1990)) and these might represent intracellular calcium storage sites. However, at present there is no substantive evidence for such subdomains in plants, principally because of a lack of markers for such a specialized compartment.

In the course of our work on the auxin-binding protein ABP1 (which has the KDEL ER retention sequence) we suggested that its apparent enrichment in parts of the ER might be associated with calcium storage domains important for intracellular signalling (Napier *et al.*, 1992). We decided to purify calreticulin in order to test this hypothesis and to assess the use of calreticulin as a marker for intracellular calcium storage sites. We have purified the protein from maize seedling ER and report on its primary structure and its intracellular distribution. We also examine what functions calreticulin might have in plant ER.

Materials and methods

Preparation of ER-enriched membrane fraction

Seeds of maize (cv. Clipper) were soaked in tap water before sowing in damp vermiculite. Seedling shoots were harvested after 5 d at 25 °C in the dark. Subsequent steps were all at 4 °C except for FPLC chromatography. Quantities given are for 100 g tissue.

Microsomal membranes were prepared as described previously (Batt *et al.*, 1976) except that the homogenization buffer was 70 mM TRIS/HCl, pH 8.0, 0.25 M sucrose, 3 mM EDTA, 10% (v/v) glycerol, 1% (w/v) bovine serum albumin, 0.5% (w/v) insoluble polyvinylpyrrolidone, 2 mM phenylmethylsulphonyl fluoride (PMSF), 4 mM dithioerythritol (DTE), and the initial centrifugation was at 10 000 *g* (rather than the previous 4 000 *g*). Microsomal membrane pellets were resuspended in 100 ml of 20 mM bis-tris-propane buffered to pH 7.0 with morpholinoethanesulphonic acid (MES), 0.25 M sucrose, 10% glycerol, 1 mM PMSF, and 1 mM DTE and re-pelleted before further resuspension in 6 ml of the same medium (Logan and Venis, 1995).

The microsomes were loaded on to sucrose steps of 20% (5.5 ml) and 30% (5 ml) w/w sucrose made up in 1 mM MES/TRIS, pH 7.2, and centrifuged for 16 h at 75 000 *g* in a Beckman SW41 rotor (Beckman, High Wycombe, UK). The ER-enriched membranes were collected with a Pasteur pipette from the 20–30% sucrose interface. A full characterization of the fractionation of maize shoot membranes using this protocol is described in Logan and Venis (1995). Membranes were either used immediately or frozen in liquid N_2 and stored at −20 °C.

Column chromatography and antibody production

ER-enriched membranes were diluted 5-fold with 20 mM morpholinopropanesulphonic acid, pH 7.2, 0.25 M sucrose, 0.5% (v/v) Nonidet-P40. After mixing they were incubated at room temperature for 15 min before centrifugation at 33 000 *g* for 30 min. The supernatant of detergent-solubilized proteins was loaded directly on to DEAE Biogel A (BioRad Labs, St Albans, UK), 6 cm × 1 cm column equilibrated in the same buffer. The column was washed to baseline and then with 0.1 M NaCl before elution with 0.3 M NaCl, all in the same buffer without Nonidet-P40.

The 0.3 M NaCl eluate was diluted 3-fold with 20 mM TRIS, pH 7.2, 1 mM EDTA and loaded on to a Mono Q HR 5/5 column (Pharmacia, Milton Keynes, UK). Proteins were eluted with a linear gradient from 0.1 to 0.7 M NaCl. Calreticulin was eluted at around 480 mM NaCl (Fig. 1). Calreticulin-containing fractions were combined and protein precipitated with trichloroacetic acid (10%, w/v, final) on ice overnight. Protein pellets were dissolved in 1 ml fresh 5 M urea (ACS grade, Sigma, Poole, UK) and made to 0.1% with trifluoroacetic acid (TFA) and loaded on to a proRPC reverse phase FPLC column (Pharmacia). Elution was with a gradient from aqueous 0.1% TFA to 0.1% TFA in acetonitrile (Fig. 2).

Microsequencing of the N-terminus was done by the Microchemical Facility, Babraham Institute, UK. Antisera were raised in rabbits as described before (Napier *et al.*, 1988) except that Titermax (Stratech, Luton, UK) was used as adjuvant. The initial immunization was with 68 μg purified protein and a boost (28 μg) in PBS was given 5 weeks later.

SDS–PAGE, immunoblotting and $^{45}Ca^{2+}$ binding

SDS–PAGE was done according to the method of Laemmli (1970) using either 10% or 12% acrylamide. Protein transfer to nitrocellulose membranes (0.2 μm, Sartorius, Epsom, UK) was done in carbonate buffer (Dunn, 1986) at 4 °C. All subsequent steps were at room temperature. Proteins were visualized with Ponceau S to check transfer efficiency, the staining pattern recorded by photocopy, before blocking in phosphate-buffered saline (PBS) plus 0.1% Tween-20 (PBSTW). Antibodies were diluted in PBSTW.

Calreticulin was monitored throughout purification with the anti-HDEL monoclonal antibody 2E7 (Napier *et al.*, 1992) and by $^{45}Ca^{2+}$ binding (Maruyama *et al.*, 1984). Briefly for the latter, the blot was washed thoroughly in 10 mM imidazole, pH 6.8, 60 mM KCl, 5 mM $MgCl_2$ before incubation for 10 min in the same plus 37 MBq $^{45}CaCl_2$ per litre. The membrane was washed with three changes of 50% aqueous ethanol in 5 min, dried and autoradiographed.

Detection for immunoblotting was with biotinylated anti-mouse (for 2E7) or anti-rabbit (for anti-calreticulin) immunoglobulins (Amersham, Aylesbury, UK) diluted 1:2000 in PBSTW and then avidin-conjugated horseradish peroxidase (Sigma) diluted 1:1000. Washes between each step were in PBSTW, but two final washes were given in PBS before development with 4-chloro-1-naphthol.

Immunofluorescence double labelling

Maize seeds (cv. LG11) were germinated on damp filter paper at 25 °C in the dark. Root tips were excised from 4-d-old seedlings under 4% paraformaldehyde in 0.1 M piperazine-*N*,*N*^{1}-bis (2-ethanesulphonic acid) 1,4-piperazine diethanesulphonic acid (PIPES) buffer, pH 6.9, and fixed for 1 h at room temperature. To permeabilize the cell walls after three washes in buffer, root tips were incubated for 15–20 min in buffer containing 1% cellulase (Sigma) and 0.17% pectinase (Sigma) which had been heat-treated at 45 °C for 10 min with 2% BSA prior to use. After further washing, root tips were gently squashed to release individual cells on to slides which had been precoated with Vectabond (Vector Laboratories, Peterborough, UK). Preparations were allowed to dry to a thin film. Cells were further permeabilized with 0.5% Triton X-100 in PIPES buffer for 10 min, followed by buffer containing 1% BSA for 10 min before incubation in primary antibody (anti-calreticulin diluted 1:2000 in the same buffer) for 1 h at room temperature. Following buffer washes containing 0.1% fish-gelatine, cells were stained with an FITC-conjugated anti-rabbit secondary antibody (Sigma), diluted 1:40. After further washes, cells were prepared for double-labelling by re-incubating in 1% BSA for 10 min. Cells were then incubated in a second primary antibody, anti-HDEL 2E7 (Napier *et al.*, 1992) as above and stained with a 1:40 dilution of rhodamine (TRITC)-conjugated anti-mouse secondary antibody (Sigma). After thorough washing, preparations were mounted in Citifluor antifade agent (City University, London) and viewed with a Zeiss Axioplan Epifluorescence Microscope using FITC and TRITC filter sets. Micrographs were recorded using Ilford HP5 photographic film.

Clones and sequencing

A λ gt11 cDNA library prepared from the mRNA of root tips of *Zea mays* var. Merit (Clontech, Cambridge Bioscience, Cambridge, UK) was screened according to the method of Huynh *et al.*, (1985). Approximately 10^6 plaques were plated using *E. coli* strain Y1090 on 22 cm square plates. Filters (Hybond-C, Amersham) were washed and blocked in TRIS-buffered saline plus 0.1% Tween-20 (TBST) before being incubated with the anti-calreticulin serum diluted in TBST. After washing, the filters were incubated in alkaline phosphatase-conjugated anti-rabbit immunoglobin (Sigma) and developed with 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium. Positive plaques were purified for further study. The DNA inserts were excised from the λ DNA on EcoRI endonuclease fragments and cloned into pBluescript (Stratagene, Cambridge, UK). The DNA was sequenced by the chain terminating method with Sequenase 2.0 (USB, Amersham) using a mixture of nested deletions and custom-designed oligonucleotide primers. Sequence comparisons were made using the Wisconsin GCG sequence analysis package.

Histone-agarose chromatography

Histone-conjugated agarose (Sigma) was incubated in 6 M urea, 50 mM dithiothreitol (DTT) at 37 °C for 15 min to denature the histone. All chromatography steps were at 4 °C. The agarose (2 ml) was then poured into an Econo-Column (BioRad) and the column was equilibrated just before use in 20 mM TRIS/HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 2.5 mM EDTA, and 1 mM EGTA.

ER-enriched membranes were solubilized by the addition of Tween-20 to 0.5% (v/v), mixing and incubation at room temperature for 10 min, then centrifuged at 13 600 *g* for 5 min (Microcentaur, MSE, Loughborough, UK) to clear. The supernatant was collected, diluted 3-fold in column buffer and loaded directly on to the histone-agarose. After washing to the baseline, the column was washed in the same buffer plus 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, before elution with 2 mM ATP in this buffer, collecting 5 ml. Final elution was with 0.1 M glycine, pH 2.5.

Maize cell cultures

The black Mexican sweet (BMS) maize cell line was obtained from Dr C. Hawes (Oxford, UK). It was cultured on basal Murashige and Skoog medium (Sigma) supplemented with 9 μM 2,4-dichlorophenoxyacetic acid and 58.5 mM sucrose. For experiments where the medium was supplemented with calcium (2 mM $CaCl_2$), EGTA (2 mM) or DTT (5 mM), the cells were grown for 10 d after subculture to the middle of the log phase of growth before test compounds were added. Cells were harvested aseptically with a 5 ml pipette, filtered (Whatman No. 1 paper, Whatman, Maidstone, UK) weighed and then freeze-dried. Total cell lysates were prepared from freeze-dried cells by adding lysis buffer (50 ml TRIS/acetic acid, pH 8.0, 0.25 M sucrose, 1 mM EDTA, 0.1 mM $MgCl_2$, 0.5 mM PMSF, 0.5% (v/v) Tween-20) at room temperature (3.3 ml per 1 g fresh weight cells). After mixing, the debris was removed by centrifugation at 20 000 *g* for 10 min and the supernatant was used immediately for SDS–PAGE or frozen in liquid N_2.

Results

Purification of calreticulin

A membrane fraction enriched in ER was collected from sucrose step gradients and luminal proteins were solubilized with the non-ionic detergent Nonidet-P40. The initial purification was from a 0.1–0.3 M NaCl cut from DEAE Biogel A (not shown) and this eluate was loaded on to Mono Q and eluted with a linear salt gradient (Fig. 1).

At least three of the more abundant ER proteins of plant cells are recognized by the anti-HDEL monoclonal 2E7 (Napier *et al.*, 1992; Fig. 1a). It seemed likely that these corresponded to the reticuloplasmins hsp 90, BiP at 70 kDa and calreticulin (at 50 kDa in maize, but between 50 and 63 kDa in mammals, Michalak *et al.*, 1992). We used $^{45}Ca^{2+}$ binding to the immobilized protein (Maruyama *et al.*, 1984) to substantiate these allocations (Fig. 1a, c). Three proteins were found to bind calcium,

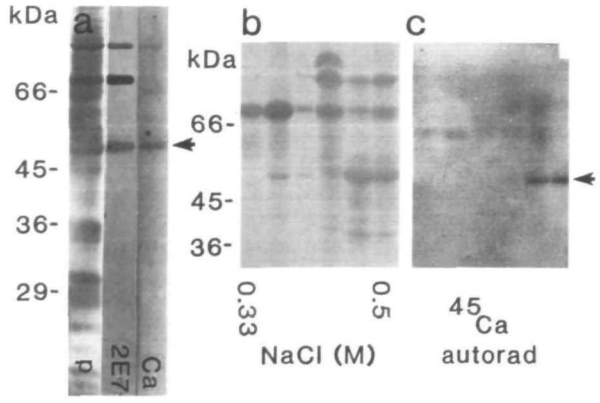

Fig. 1. Purification of calreticulin on Mono Q and calcium binding overlays. (a) Detergent-solubilized ER proteins stained for protein (P), with 2E7 and overlaid with $^{45}Ca^{2+}$. The calreticulin band is marked (►). BiP does not correspond to a calcium-binding band, but hsp 90 appears to bind calcium weakly. (b) Proteins eluted by a linear gradient of NaCl (0.3–0.5 M shown). The blot was stained for protein with Ponceau S. (c) The same fractions overlaid with $^{45}CaCl_2$ for calcium-binding proteins.

but strong binding was found only with the 50 kDa polypeptide which was also recognized by 2E7. Consequently, we chose mobility on SDS–PAGE and anti-HDEL blotting to follow routinely the putative 50 kDa calreticulin through purification. It eluted from Mono Q at around 480 mM NaCl (Fig. 1b, c).

After precipitation of combined calreticulin-containing fractions with TCA, the pellets were dissolved in urea + 0.1% TFA and loaded on to a reverse phase (mixed C_1 and C_8 alkyl chains, proRPC) column. Elution was with a gradient to acetonitrile (Fig. 2). Calreticulin eluted predominantly over two fractions, the second of which was free from all but one other polypeptide of slightly higher M_r. Although separations on hydroxapatite, phenyl-Sepharose and Mono Q in the presence of zinc (calreticulin also binds zinc, which induces a conformational change in the protein and changes its chromatography, Heilmann *et al.*, 1993) have been tested we have been unable to separate these two polypeptides (data not shown). Additionally, both are recognized by 2E7 (Figs 1, 7) and both are stained by concanavalin A suggesting that both are N-glycosylated (not shown). A similar band is seen in human liver calreticulin preparations (Heilmann *et al.*, 1993).

The N-terminal amino acid sequence was determined for the major, putative calreticulin band (Fig. 4). It was found to be strongly homologous with a range of calreticulin sequences from mammals, *Xenopus* and *Caenorhabditis* as well as sequences from spinach (Menegazzi *et al.*, 1993). The minor band was also sequenced, but while some residues were identical, some could not be assigned unequivocally and insufficient data were obtained to allow matching. On the basis of the identity of the major band, and the likelihood that the minor band was either an isoform or glycoform, these purified calreticulin fractions were used to raise an antiserum.

The antiserum was tested against detergent-solubilized crude microsomal fractions from a range of plants (Fig. 3). In each case calreticulin is detected strongly and a subsidiary, slightly higher M_r band is also recognized, presumably representing the upper, minor polypeptide in the calreticulin fractions used for immunization. In dicotyledonous plants the two proteins have a higher M_r than in monocotyledonous species, but the size difference

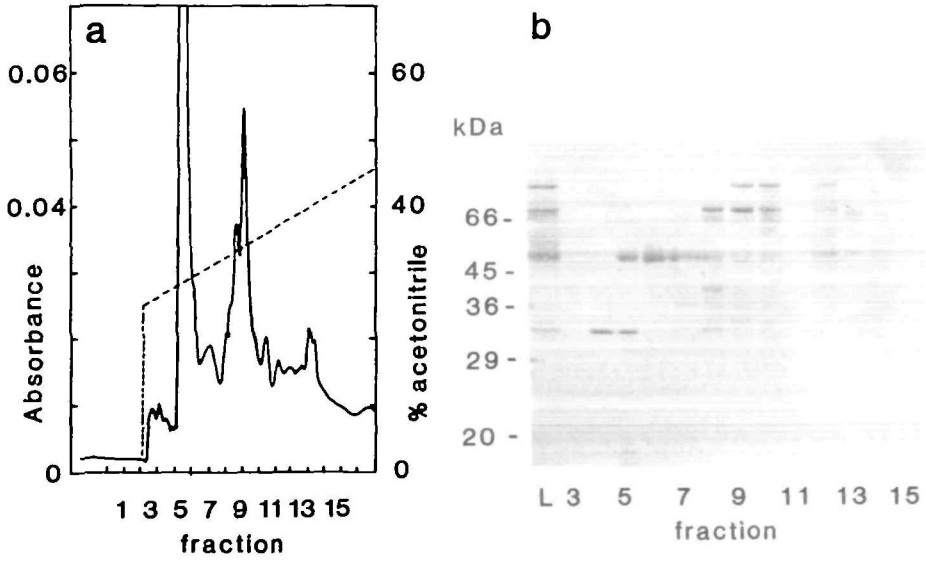

Fig. 2. Purification of calreticulin by reverse phase. (a) Proteins from DEAE chromatography eluted by a gradient of acetonitrile. (b) Fractions stained with Ponceau S. L = the DEAE eluate used to load the proRPC column. For routine purifications, calreticulin-containing fractions from Mono Q (rather than the initial DEAE salt cut shown here) would be loaded on to the proRPC column.

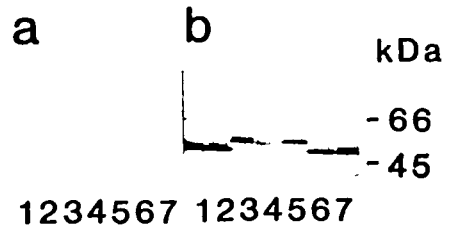

Fig. 3. Monocotyledonous and dicotyledonous calreticulins. Immunoblot of detergent-solubilized proteins from crude microsomes of (1) maize; (2) barnyard grass; (3) sugar beet; (4) cotton; (5) mung bean; (6) sorghum; (7) wheat. (a) Pre-immune, (b) anti-calreticulin.

between the major calreticulin band and the other remains unchanged.

Selection and sequencing of calreticulin cDNAs

The antibody was used to screen an expression library of mRNAs derived from maize root tips. Twenty positive plaques were selected, rescreened to obtain pure clones, and several inserts which carried a complete coding sequence were isolated. The full coding sequence (Fig. 4a; EMBL accession number Z46772) was located on a 2.0 kb fragment. The predicted amino acid sequence has been compared to sequences from human, *Hordeum, Drosophila*, rabbit and *Caenorhabditis* (Fig. 4b). The first 25 residues show characteristics common to other signal peptide sequences (von Heijne, 1984), with possible cleavage sites after serine (−7) and glycine (−1) (Fig. 4a). Our N-terminal sequence shows that the latter residue is used to yield a mature protein of 396 residues with a predicted molecular weight of 47 500. The sequence shows one possible site for *N*-glycosylation (residues 31–33) and ends with the tetrapeptide HDEL, which is likely to confer retention in the ER. In addition, a highly conserved putative nuclear targeting signal (Raikhel, 1992) is found between residues 185–193. The amino acid sequence shows 96% identity with *Hordeum* (Chen *et al.*, 1994) and 55% with animal calreticulins (Fig. 4b). Calreticulin sequences have been divided into three functional regions (Michalak *et al.*, 1992) and the maize sequence fits the same domain pattern (indicated on Fig. 4b). Sequence conservation (boxed residues) is particularly marked over the P region, so named because it is rich in proline residues. It is within this region that there are three conserved repeats of the DP(E/D)(A/D)XKP-EDWD(D/E) motif, although in maize (and *Hordeum*) the second is varied to KPEGYDD. There are also three repeats of the motif GXW. Both of these motifs are highly conserved in calreticulin sequences and in calnexins (Michalak *et al.*, 1992; Huang *et al.*, 1993). We have called the locus coding for calreticulin *crt1*.

Immunofluorescence and intracellular distribution

Double-label, indirect immunofluorescence was used to investigate the intracellular distribution of calreticulin in maize root cells. Calreticulin was seen to be located only within the ER and nuclear envelope and was not found in the nucleus (Fig. 5a). The fluorescence pattern is identical to that of 2E7 (Fig. 5b) which was used to report on all HDEL proteins, but of which BiP is the most abundant (Figs 6, 7). No evidence was found for enrichment of calreticulin in subdomains of the ER in these root cells.

Regulation of calreticulin levels

In order to examine whether or not the plant cell responds to changes in extracellular calcium by regulating its intracellular storage capability, we raised or reduced calcium levels in the medium of BMS maize cell suspensions. Cells were tested in the mid-part of their logarithmic growth phase and grown for up to 60 h with each treatment (Fig. 6). Calreticulin levels were determined from immunoblots of total cell lysates, and 2E7 was used (staining BiP primarily) for comparison. No changes in calreticulin (or BiP) levels were observed, either in the short-term (30 min, not shown) or longer-term (48 h).

Calreticulin binds to denatured protein

In addition to its role in calcium storage, there is evidence that mammalian calreticulin is a molecular chaperone (Michalak *et al.*, 1992). We have used immobilized denatured protein (histones) to examine the possibility that maize calreticulin is a chaperone (Nigam *et al.*, 1994). Histone immobilized on agarose was denatured with urea and DTT and loaded with detergent-solubilized ER proteins. After thorough washing, chaperones were eluted specifically with ATP (Fig. 7). All the HDEL proteins were eluted, BiP and hsp 90 (identity confirmed here with the anti-ER-hsp 90 antiserum of Schröder *et al.*, 1993) and calreticulin. Some residual protein is eluted by low pH in a subsequent wash, but this fraction contains the same spectrum of proteins as the ATP eluate. Calcium ions were routinely present in the column wash prior to addition of ATP and during ATP elution (see Materials and methods), but calcium was found to be unnecessary for elution. Neither did calcium induce elution in the absence of ATP at concentrations up to 5 mM (not shown).

The nucleoside phosphate specificity differs between chaperones (Fig. 8). A series of nucleoside di- and triphosphates was used to elute sequentially from one column. Diphosphates eluted weakly, although there was some preference of calreticulin for GDP over other diphosphates. Calreticulin was eluted by GTP about as effectively as by ATP (compare to Fig. 7), although BiP showed greater specificity for ATP.

In order to examine the possibility that there might be co-ordinate transcriptional regulation of reticuloplasmins (McCauliffe *et al.*, 1992), we tested cell suspensions for

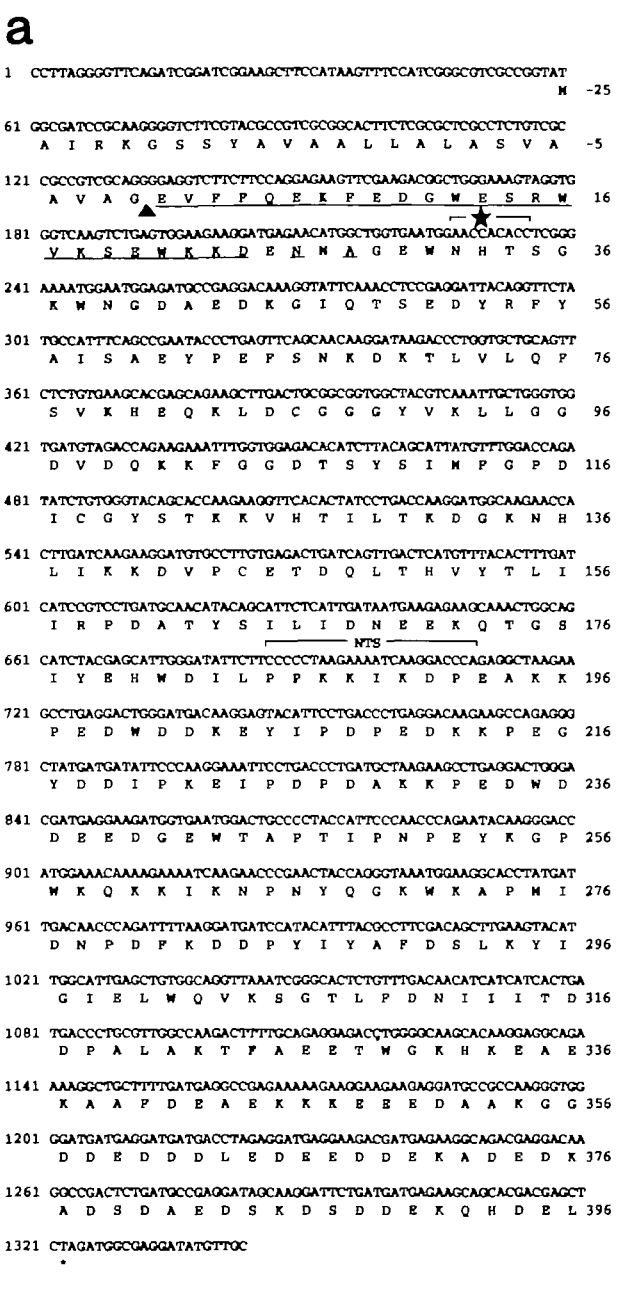

Fig. 4. Sequence analysis of maize calreticulin. (a) The nucleotide and deduced amino acid sequences. Deduced amino acid residues are shown in the single letter notation under the nucleotide sequence. Numbering in the right margin refers to the amino acid sequence (in which we number the first residue of the mature, purified protein (1) and in the left margin the nucleotide sequence. The arrowhead indicates the site of signal peptide cleavage and the *N*-terminal amino acid sequence determined from purified calreticulin is underlined. The star represents the single possible *N*-glycosylation site and the bracket represents the putative nuclear targeting sequence (NTS). (b) Comparison of deduced amino acid sequences of calreticulin from *Zea* (this work), *Hordeum* (Chen *et al.*, 1994), *Drosophila* (Database Accession number A37158), rabbit (Fliegel *et al.*, 1989) and *Caenorhabditis* (Database Accession number S25851). The N, P and C regions are distinguished by shading. The conserved repeat motifs are indicated by arrows.

their response to conditions under which nascent polypeptides were unable to fold correctly. When applied *in vivo*, the reducing agent DTT prevents the formation of disulphide bridges within and between proteins in the ER (Braakman *et al.*, 1992; Tatu *et al.*, 1993). Consequently, there is a build-up of unfolded, or incorrectly folded proteins. Under these conditions, in maize cell suspensions, the well-characterized ER chaperone BiP was strongly up-regulated (Fig. 6c). Calreticulin levels remained unchanged.

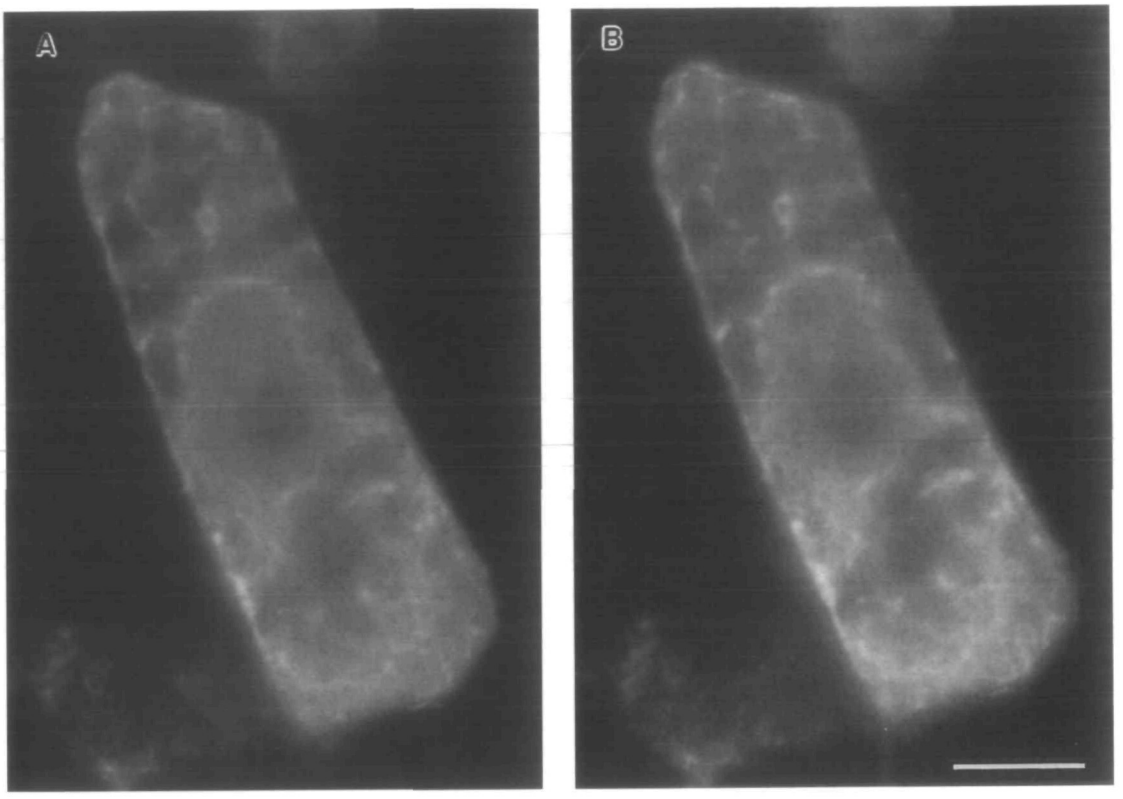

Fig. 5. Intracellular distribution of calreticulin. Double immunofluorescence labelling of maize root cells showing that the staining pattern with anti-calreticulin (a) co-localizes with the staining pattern obtained with monoclonal antibody 2E7 (anti-HDEL), an ER marker antibody in plant cells (b). Scale bar = 10 μm.

Discussion

We have shown that the ER luminal calcium-binding protein calreticulin can be purified from maize seedling tissue by ion-exchange and reverse-phase chromatography. As an initial step a salt cut from DEAE was used. Hydroxyapatite can be used equally well, with calreticulin eluting between 0.1 and 0.25 M phosphate (not shown). High resolution ion-exchange and reverse-phase chromatography yielded a fraction highly enriched in calreticulin together with an additional minor band of slightly higher M_r (Fig. 2). The identity of the major band was confirmed as calreticulin by N-terminal sequencing, but we failed to identify the minor band with any certainty. Similar doublet patterns for calreticulin preparations have been observed before, with human calreticulin for example (Heilmann *et al.*, 1993) and, in mammals, different forms of calreticulin seem to be expressed in different tissues indicating a range of isoforms (Michalak *et al.*, 1992). Two distinct clones of calreticulin have been isolated from *Hordeum* (Chen *et al.*, 1994) and so it seems likely that the minor band co-purifying with calreticulin is an isoform of calreticulin.

The antiserum raised to maize calreticulin was found to be specific for the 50 kDa calreticulin band in whole cell lysates of suspension-cultured maize cells (Fig. 6). It also recognized calreticulin (and the minor upper band) in all species tested, both monocotyledonous and dicotyledonous (Fig. 3). In passing, we point out that we have tested a range of antisera raised against animal calreticulins and found them to show little or no cross-reactivity to the plant homologue. They were tested by immunoblotting membrane preparations, immunofluorescence and screening expression libraries and were found to be of no practical use for our experiments. These findings are in contrast to two previous reports (Allen and Tiwari, 1991; Zwialek *et al.*, 1993), but there has yet to be confirmation of these other, preliminary, observations.

We have used our antiserum to screen a λ gt11 expression library of maize mRNAs and, of the 20 clones selected, all showed considerable homology to all or part of other calreticulin sequences. As for *Hordeum* calreticulin (Chen *et al.* 1994), the coding sequence of the mature maize calreticulin (signal peptide cleaved) shows high identity to calreticulins from diverse sources. The degree of identity differs between the three domains into which the protein has been divided (Michalak *et al.*, 1992). In the N region (residues 1–185), the region found to show most variation among animal calreticulins, identity was 45% although key residues are conserved (such as cysteine 118 and cysteine 144, between which there is a disulphide

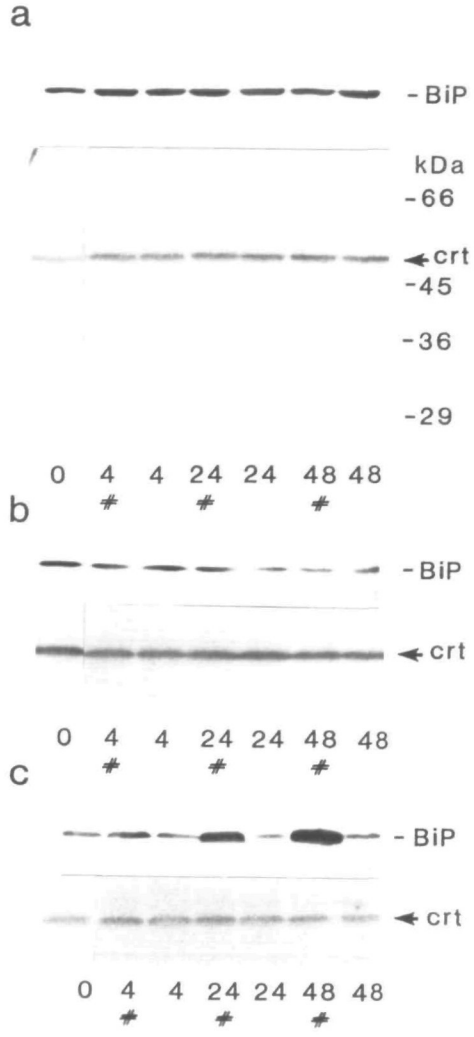

Fig. 6. Regulation of maize calreticulin by calcium and stress. Immunoblots stained with either anti-calreticulin (lower panel) or 2E7 (upper panel). At these loadings, the only HDEL protein labelled (at 70 kDa) is BiP. Each track was loaded with cell lysate from 15 mg fresh weight of cells. (a) Cells grown with (#) or without additional 2 mM $CaCl_2$, (b) with (#) or without 2 mM EGTA, and (c) with (#) or without 5 mM DTT. Treatments were started during log phase growth ($t=0$) and samples taken after 4, 24 and 48 h from both treated (#) and untreated flasks.

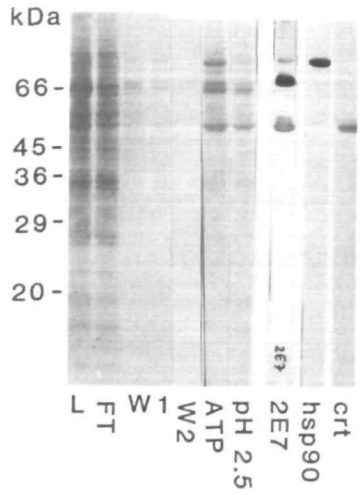

Fig. 7. Calreticulin binds to denatured histone. Detergent-solubilized ER proteins (L) were passed through a column of denatured histone linked to agarose. The column was washed in loading buffer (W1) followed by loading buffer + 2 mM $MgCl_2$ + 0.5 mM $CaCl_2$ (W2). Chaperones were eluted with ATP followed by glycine (pH 2.5). Parallel ATP-eluted samples were stained for protein, HDEL proteins (with 2E7), hsp 90, and calreticulin (crt).

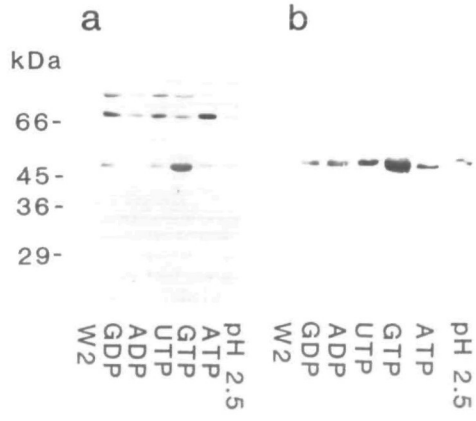

Fig. 8. Nucleoside phosphate specificity for elution from denatured histone-agarose. A column prepared and loaded as in Fig. 7 was eluted sequentially with nucleoside phosphates as shown (all 2 mM in buffer with 2 mM Mg^{2+} and 0.5 mM Ca^{2+}), (a) stained with 2E7 and (b) stained with anti-calreticulin.

bond; Matsuoka *et al.*, 1994). Identity was 65% in the P region (residues 186–297), the region in which the high affinity calcium binding site is found. The C region is rich in acidic residues and contributes the many low affinity calcium binding sites to calreticulin. Animal calreticulin C regions comprise 41% aspartic and glutamic acid residues which compares well with 38% in maize. The relatively low identity found in this region (45%) is largely explained by the preponderance of aspartate residues in plants and glutamate residues in animals, suggesting that the acidic properties of the region are conserved rather than the individual amino acids.

Up-regulation of calreticulin as well as BiP and hsp 90 was reported in gibberellin-stimulated barley aleurone cells, coincident with the rise in α-amylase secretion (Denecke *et al.*, 1995). This corresponds with proliferation of ER in these cells (Evins and Varner, 1971). The clones of Chen *et al.* (1994) were used to show that calreticulin is dramatically up-regulated after pollination in *Hordeum*, in either embryo or embryo and ovary tissue. This is in accord with observations on the transcriptional regulation of mammalian calreticulins, which are up-regulated in proliferating cells and are thought to play a role in cell proliferation (Michalak *et al.*, 1992). Promoter analysis (of mammalian calreticulin genes) identified regulatory sites shared with genes for other reticulo-

plasmins, BiP, grp 94 and PDI and these genes seem to be co-ordinately regulated, with high expression in rapidly dividing cells (McCauliffe *et al.*, 1992). No evidence for co-ordinate regulation in response to stress was found for maize BiP and calreticulin (Fig. 6c). The reducing agent DTT has been shown to inhibit formation of disulphide bridges within and between proteins in the ER *in vivo* (Braakman *et al.*, 1992; Tatu *et al.*, 1993), leading to a build-up of unfolded or misfolded proteins. The chaperone BiP is strongly induced under these conditions (Fig. 6c), but calreticulin levels were not measurably changed. Similar lack of co-ordinate regulation was reported for tobacco calreticulin (Denecke *et al.*, 1995). Either co-ordinate regulation of reticuloplasmins is not a feature of plant cells, or it is possible that co-ordinate regulation of reticuloplasmins is specific for proliferating cells (and proliferation of the whole ER in aleurone might be similar to this), not for response to stress, and we have yet to examine this possibility.

Even though there was no apparent accumulation of calreticulin in response to rises in the level of unfolded proteins in the ER, maize calreticulin did behave *in vitro* like the chaperones BiP and hsp 90. Calreticulin and the recognized ER chaperones BiP and hsp 90 all bound to denatured histone and were released specifically by nucleoside triphosphates. For calreticulin, elution was most efficient with either GTP or ATP (but not UTP; Fig. 8). Mammalian calreticulin was similarly eluted by ATP, although it was reported that its elution was calcium-dependent (Nigam *et al.*, 1994). Maize calreticulin was found to show no such dependence.

Elution by nucleoside triphosphates is, however, curious because no recognized ATP binding site has been identified in calreticulin (Michalak *et al.*, 1992). Nigam *et al.* (1994) point out that there is a putative ATP binding site within the N-domain, but they were unable to label the protein with photoactive ATP. It is possible that calreticulin binds to denatured protein (histone) as part of a complex with BiP and hsp 90. Subsequent hydrolysis of ATP by either BiP or hsp 90 might then induce dissociation of all components of the complex. No evidence of preformed chaperone complexes was found using size exclusion chromatography, calreticulin eluting with a monomeric size of between 60–80 kDa (not shown). However, a complex might form around the denatured protein substrate, and further work will be necessary to explain these observations. Nevertheless, we did find nucleoside specificity for elution from histone (Fig. 8) and despite the uncertainty over the mechanism of calreticulin release, the results suggest strongly that maize calreticulin behaves in a manner consistent with it being a molecular chaperone. However, we have not yet attempted to show that maize calreticulin assists in protein folding, a fundamental property of chaperones.

We have mentioned that we used the C-terminal HDEL targeting sequence (as well as calcium binding) to follow calreticulin through purification. The sequence data also indicate an N-terminal signal peptide (Fig. 4). Immunofluorescence confirms that calreticulin is confined to the ER and nuclear envelope of maize root cells (Fig. 5) and in cells of tobacco seeds (Denecke *et al.*, 1995). Double labelling demonstrates that it is distributed throughout the ER with exactly the distribution of the total HDEL protein complement (principally reflecting the distribution of BiP, see Figs 6 and 7; Oliver, Venis and Napier, unpublished). We found no evidence for subdomains enriched in calreticulin. Such subdomains have been described in animal cells, although they do seem to be cell-type-specific. Calreticulin has been used as a marker for calcium-rich storage domains (along with the ER Ca^{2+}-ATPase), in platelets and a rat myogenic cell line (Arber *et al.*, 1992). However, in rat cerebellum calreticulin was found throughout the ER, as was BiP and calnexin, even though IP_3 receptors were concentrated in specialized subdomains (Nori *et al.*, 1993). The maize auxin-binding protein ABP1 appeared to have a restricted distribution within the ER in maize root cells (Napier *et al.*, 1992) and it was suggested that these might correspond to cortical/calcium storage ER domains. We have shown that maize calreticulin does bind calcium (Fig. 1), but with no evidence for calreticulin-enriched areas, either other indicators of calcium storage domains must be sought or other explanations found for the particular distribution of ABP1.

We have examined our immunofluorescently-labelled root cells by confocal scanning laser microscopy for any label which might be localized in the nucleus (not shown), but have not detected any. There is evidence to suggest that (in mammalian cells) calreticulin regulates transcription by binding to nuclear hormone receptors (Burns *et al.*, 1994; Dedhar *et al.*, 1994; Dedhar, 1994) and some evidence for nuclear distribution (in addition to ER) has been found (Opas *et al.*, 1991). In plants, calreticulin is up-regulated in embryonic, proliferating cells (Chen *et al.*, 1994) and it will be interesting to investigate the intracellular distribution of calreticulin in these or in cells derived specifically from the root meristem (it is difficult to tell the origin of cells in root squash preparations) and to test whether plant calreticulin might regulate transcription in addition to its role in calcium storage (Fig. 1) and its putative role as a chaperone (Figs 7, 8).

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