BiP and Calreticulin Form an Abundant Complex That Is Independent of Endoplasmic Reticulum Stress

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BiP is found in association with calreticulin, both in the presence and absence of endoplasmic reticulum stress. Although the BiP–calreticulin complex can be disrupted by ATP, several properties suggest that the calreticulin associated with BiP is neither unfolded nor partially or improperly folded. (1) The complex is stable in vivo and does not dissociate during 8 hr of chase. (2) When present in the complex, calreticulin masks epitopes at the C terminus of BiP that are not masked when BiP is bound to an assembly-defective protein. And (3) overproduction of calreticulin does not lead to the recruitment of more BiP into complexes with calreticulin. The BiP–calreticulin complex can be disrupted by low pH but not by divalent cation chelators. When the endoplasmic reticulum retention signal of BiP is removed, complex formation with calreticulin still occurs, and this explains the poor secretion of the truncated molecule. Gel filtration experiments showed that BiP and calreticulin are present in distinct high molecular weight complexes in which both molecules interact with each other. The possible functions of this complex are discussed.

INTRODUCTION

The endoplasmic reticulum (ER) is the primary organelle of the endomembrane system and is responsible for the biosynthesis and maturation of proteins that are destined for secretion, for the plasma membrane, and for transport to various organelles of the endocytic and exocytic pathways (reviewed in Palade, 1975; Helenius et al., 1992; reviewed for plants in Vitale et al., 1993). In addition to nascent polypeptides and newly synthesized proteins, the ER lumen also contains a large and abundant family of soluble resident proteins known as the reticuloplasmins (Koch, 1987; reviewed for plants in Denecke, 1996). Several reticuloplasmins have been identified in plants, including the binding protein BiP (Denecke et al., 1991; Fontes et al., 1991; Anderson et al., 1994), calreticulin (Chen et al., 1994; Denecke et al., 1995; Dresselhaus et al., 1996), protein disulfide isomerase (PDI; Shorrosh and Dixon, 1991), and endoplasmin (Walther-Larsen et al., 1993; Denecke et al., 1993, 1995). Endoplasmin has homology with the cytosolic heat shock protein Hsp90 family, but no definitive function has been proposed. PDI catalyzes the formation of disulfide bonds, a process restricted to the ER lumen, hence the absence of cytosolic homologs of PDI.

The best-characterized ER resident protein is BiP (Munro and Pelham, 1986). BiP belongs to the Hsp70 family of molecular chaperones. This family has members in every compartment of the cell in which protein folding occurs. These proteins contain an N-terminal ATPase domain and a C-terminal polypeptide binding domain (Hendrick and Hartl, 1993). Hsp70 members have in vitro affinity for peptides rich in hydrophobic residues, which are likely to be exposed in intermediates of structural maturation or in malfolded polypeptides, although the preference for amino acid patterns is member specific (Fourie et al., 1994).

In common with the other reticuloplasmins, BiP has both a signal sequence and a C-terminal ER retention signal (Vitale et al., 1993). Genetic complementation experiments have shown that plant BiPs perform the same function as yeast and mammalian BiPs (Denecke et al., 1991). Experiments with the homotrimERIC bean storage protein phaseolin have shown that BiP associates with the monomeric protein before its assembly into trimers (Vitale et al., 1995) and that...
assembly-defective phaseolin associates more extensively with BiP than does the wild-type form (Pedrazzini et al., 1994, 1997).

Calreticulin is a highly conserved and ubiquitous protein that is found in the ER and nuclear envelope (Denecke et al., 1995; reviewed in Michalak, 1996). The glycan structure of spinach calreticulin has been determined (Navazio et al., 1996), and the same protein has been found to be specifically phosphorylated by protein kinase CK2 (Baldan et al., 1996). Many functions have been proposed for calreticulin. These include roles in Ca$^{2+}$ binding (Michalak et al., 1992), Ca$^{2+}$ signaling (Mery et al., 1996), cell adhesion (Coppolino et al., 1997), and gene expression (Michalak et al., 1996). In addition, a possible function as a molecular chaperone has been proposed (Denecke et al., 1995; Nauseef et al., 1995).

Evidence in support of a chaperone function for calreticulin comes in part from the fact that calreticulin has significant sequence homology with calnexin, an ER type I transmembrane protein, which is reported to be a chaperone (Rajagopalan et al., 1994; Hebert et al., 1996; Vassilakos et al., 1996; Tatu and Helenius, 1997). Tobacco calreticulin is present in protein complexes in vivo in a stress- and ATP-dependent manner (Denecke et al., 1995). Similar work with mammalian cells has also implicated calreticulin as a molecular chaperone (Nauseef et al., 1995; Hebert et al., 1996; Otteken and Moss, 1996).

Both calreticulin and calnexin demonstrate lectin-like activity and are able to bind to the monoglucosylated Asn-linked glycans of glycoproteins (Hammond et al., 1994; Hebert et al., 1995). The existing model for substrate binding and release by these two proteins is dependent on the activity of enzymes that are responsible for the glucose trimming and reglucosylation of high-mannose Asn-linked glycans (Hebert et al., 1995; Rodan et al., 1996). When cotranslationally transferred to the growing polypeptides, these glycans have the structure Glc$_3$Man$_9$GlcNAc$_2$. Soon after transfer, the three Glc residues are removed by two ER-located enzymes. Glucosidase I removes the most external glucose, and glucosidase II successively removes the two remaining glucose residues. UDP-glucose:glycoprotein glucosyltransferase is responsible for the reglucosylation of nonglucosylated glycans to monoglucosylated forms, turning them back into ligands for calreticulin and calnexin. The biological meaning of this process is not well understood; however, reglucosylation occurs only on glycopolypeptides that have not reached their final conformation, suggesting that the calnexin-calreticulin/glucosyltransferase system monitors and aids in structural maturation and contributes to the ER retention of immature or defective proteins (Sousa and Parodi, 1995).

In this study, we show that the previously unidentified 75-kD protein that was found in complexes with calreticulin in both the presence and absence of stress (Denecke et al., 1995) is the lumenal binding protein BiP. The results from the experiments described in this study helped us to characterize this novel protein complex.

RESULTS

BiP and Calreticulin Form a Stable ATP-Dependent Complex

The most abundant protein complex formed by calreticulin involved a 75-kD protein that was associated even in the absence of stress (Denecke et al., 1995). To determine whether this protein is BiP, tobacco mesophyll protoplasts were labeled for 3 hr with a mixture of $^{35}$S-Met and $^{35}$S-Cys under normal conditions or heat shock treatment, as described previously (Denecke et al., 1995). After homogenization, the extract was immunoprecipitated with the anti-calreticulin antiserum, and the pellet was washed with ATP followed by immunoprecipitation from the supernatant with anti-BiP antibodies. Figure 1A shows that the 75-kD protein released by ATP is recognized by anti-BiP antibodies, confirming its

![Figure 1](image-url)
identity. Neither ADP nor AMP caused complex dissociation (Figure 1B). GTP did not result in efficient release of BiP from calreticulin, suggesting that the association is ATP specific (data not shown).

The observed nucleotide specificity corresponds to that for BiP-unfolded protein complexes. Because BiP is not a glycoprotein, association of calreticulin cannot be due to the recognized lectin function of the latter (Hebert et al., 1995; Spiro et al., 1996). Under heat shock treatment, very little calreticulin was synthesized during the pulse labeling (detectable only after very long exposure); therefore, the labeled BiP immunoprecipitated with anti-calreticulin antibodies must have coprecipitated with unlabeled calreticulin (Figure 1A). This shows that newly synthesized BiP becomes rapidly associated with preexisting calreticulin that was synthesized before the pulse labeling. This observation together with the abundance of the complex in the absence of stress make it unlikely that BiP is associated with unfolded calreticulin.

To exclude the possibility that the association of BiP and calreticulin is only short lived and due to the presence of transiently unfolded calreticulin, we performed pulse–chase experiments. Figure 2A shows that the complex is stable between 0 and 8 hr after the chase, with very little protein degradation and an almost constant BiP/calreticulin ratio. This strongly suggests that BiP is associated with correctly folded calreticulin and not with incompletely or improperly folded calreticulin, the latter of which would be destined for degradation. Figure 2B shows that with longer exposure of fluorographs, it is possible to see the transient interaction of BiP with numerous newly synthesized polypeptides of various sizes. Eight hours after the chase, virtually all of the BiP-coselected polypeptides had been released from the chaperone, most probably because they had acquired the correct conformation (Figure 2B, cf. lanes 1 and 5; the polypeptides other than BiP in lane 5 are nonspecific contaminants, see lanes 4 and 6). Prior depletion of calreticulin in the extract gave a very similar pattern (Figure 2B, cf. lanes 2 and 6). However, an identical amount of BiP coprecipitated with calreticulin at 0 or 8 hr after the chase (Figure 2B, cf. lanes 3 and 7), showing that BiP and calreticulin were still interacting with each other after BiP had released the newly synthesized polypeptides.

Surprisingly, immunoprecipitation with the anti-BiP antiserum did not lead to coprecipitation of calreticulin (Figure 2B, lanes 1 and 2, and 5 and 6), suggesting that the BiP-calreticulin complex is not recognized by the BiP antibodies. This could be explained by the fact that the serum had been generated against a BiP fusion protein carrying only the C-terminal 113 amino acids of BiP. In the complex, these epitopes were probably masked by calreticulin, hence the failure of the serum to recognize the complex. BiP depletion from the extract using an excess of BiP antibodies in immunoprecipitations still revealed a portion of BiP when coimmunoprecipitated with the anti-calreticulin antiserum (data not shown), confirming that the BiP-calreticulin complex is not recognized by the BiP antiserum.
BIP Interacts Differently with Calreticulin and Assembly-Defective Phaseolin

We next wanted to compare the binding of BIP to calreticulin with the binding of BIP to structurally defective proteins. For this reason, we used transgenic plants overproducing a mutant phaseolin that remains permanently monomeric (Δ363) and interacts extensively with BIP (Pedrazzini et al., 1994, 1997). Protoplasts were pulse labeled for 1 hr, and immunoprecipitations were performed with anti-phaseolin, anti-BIP, or anti-calreticulin sera. Figure 3 confirms that both anti-phaseolin and BIP antisera recognized the BIP–phaseolin complex and that this association is ATP sensitive (Pedrazzini et al., 1994). Calreticulin was not found in a complex with phaseolin and was not coprecipitated when anti-BIP antiserum was used (as shown in Figure 2B). In contrast, anti-calreticulin antiserum coprecipitated BIP but not phaseolin. Therefore, BIP bound to calreticulin and BIP associated with assembly-defective phaseolin represent different subsets of the BIP pool. The BIP–calreticulin interaction is distinct from the BIP-defective protein interaction despite its ATP dependence, because the C-terminal epitopes of BIP are only masked by calreticulin, not by phaseolin. The amount of newly synthesized BIP associated with calreticulin was greater than that associated with Δ363 phaseolin (Figure 3, cf. BIP in lanes 3 and 6), which is the major ligand that can be immunoselected with BIP, using the anti-BIP antiserum (Figure 3, lane 2; see Pedrazzini et al., 1997).

Recruitment of BIP into the Complex Is Unaffected by Altered Calreticulin Levels

We analyzed cell extracts from transgenic plants overproducing calreticulin (A. Crofts and J. Denecke, unpublished results). The rationale behind this experiment was the fact that overproduction should lead to the steady state presence of a higher number of calreticulin polypeptides that are in the process of folding. A pulse-labeling experiment was performed with the cells from control plants and calreticulin overproducers exhibiting >20-fold higher calreticulin protein levels. Figure 5 shows that calreticulin overexpression leads to an increase in noncomplexed calreticulin. If equal amounts of calreticulin were used in immunoprecipitation experiments, associated BIP became barely detectable. The binding of BIP to calreticulin is therefore independent of the number of calreticulin molecules and represents further evidence suggesting that the calreticulin complexed to BIP is not malfolded or in the process of folding. In contrast, immunoprecipitation of extracts from BIP-overexpressing plants (N. Leborgne-Castel and J. Denecke, manuscript in prepara-

Complex Stability Is Reduced by Low pH but Not by Divalent Cation Chelators

Additional experiments were conducted to establish the conditions of BIP release from calreticulin. The pH of the ER lumen is probably close to neutrality (Marquardt and Helenius, 1992), creating an environment in which the numerous acidic residues near the C termini of BIP and calreticulin are negatively charged. This could lead to the association of proteins by divalent cation bridges, which has been suggested to occur because of the high concentration of calcium ions in the ER lumen (Macer and Koch, 1988). Figure 4A shows that the pH does indeed affect the amount of BIP associated with calreticulin and that lower pH leads to the release of BIP in vitro.

Figure 4B shows that increasing the concentration of the general divalent cation chelator EDTA from 1 mM (the normal concentration in our homogenization and immunoprecipitation buffers) to 10 mM did not alter the amount of BIP coprecipitated with calreticulin. The addition of the calcium-specific chelator EGTA, in the presence of 1 mM EDTA, also had no effect (Figure 4B). EDTA was maintained in the buffer to inhibit the ATPase activity of BIP, which would be stimulated by EGTA (Suzuki et al., 1991). Our results indicate that divalent cations are not involved in the interaction of BIP and calreticulin per se.

Figure 3. Interaction of BIP with Calreticulin and with Unassembled Phaseolin.

Protoplasts from the leaves of transgenic plants expressing Δ363 phaseolin were labeled in vivo for 1 hr. Immunoprecipitation was performed with anti-phaseolin, anti-BIP, or anti-calreticulin antibodies, as indicated. Immunoprecipitates were then washed with BIP release buffer alone (−) or supplemented with 3 mM ATP (+). Molecular mass markers are given at right in kilodaltons. At left, the positions of BIP, calreticulin (Cal), and phaseolin (Phas) are indicated. Phaseolin was synthesized in two glycoforms having molecular masses of 46 and 43 kD. Note that more BIP coprecipitates with calreticulin than with the overproduced assembly-defective phaseolin.
tion) demonstrates a further recruitment of BiP to the complex with calreticulin, as observed by a higher BiP/calreticulin ratio (Figure 5A).

In BiP overproducers, we also observed a lower molecular weight form of calreticulin that was absent in untransformed plants or plants overproducing calreticulin. This lower molecular weight form shows resistance to endoglycosidase H, an enzyme that removes high-mannose glycans but not Golgi-modified, complex glycans (Figure 5B). It is either a different calreticulin isoform devoid of glycans or a glycosylated form that has been modified in the Golgi apparatus to acquire complex structures, resulting in a visible shift when subjected to SDS-PAGE. Under normal conditions, calreticulin is only present with two glycans of the high-mannose type (A. Crofts and J. Denecke, unpublished results). The fact that BiP overproduction influences calreticulin in such a manner provides additional evidence for the interaction of BiP with calreticulin in vivo. Most importantly, the results presented in Figure 5 indicate that it is the level of BiP and not the level of calreticulin that influences the formation of the complex.

The ER Retention Signal of BiP Is Not Required for Complex Formation with Calreticulin

The fact that the C terminus of BiP is masked by calreticulin prompted us to test whether the ER retention signal is required.
for complex formation. Transgenic tobacco plants producing a truncated BiP molecule lacking the tetrapeptide HDEL and the four adjacent amino acids (to be described in N. Leborgne-Castel and J. Denecke, manuscript in preparation) were used for in vivo labeling experiments. Extracts were immunoprecipitated with either anti-BiP or anti-calreticulin antibodies (Figure 6). Truncated BiP can be separated by SDS-PAGE from wild-type BiP and recognized by a small shift in the molecular weight (Figure 6, lane 2). Both forms of BiP bind equally well to calreticulin (Figure 6, cf. lanes 2 and 3), indicating that the extreme C terminus of BiP is not required for association with calreticulin. Previous experiments with truncated BiP showed that the intracellular half-life of the truncated BiP is barely distinguishable from that of wild-type BiP (N. Leborgne-Castel and J. Denecke, manuscript in preparation). It is thus possible that the HDEL-less BiP is retained in the ER by interaction with HDEL-containing calreticulin, providing further evidence that the two molecules interact in vivo.

BiP and Calreticulin Form Large Complexes

In all previous experiments, only the portion of BiP and calreticulin synthesized during the pulse labeling was detectable. The signals obtained would be influenced by the number of methionine and cysteine residues in either molecule as well as the protein synthesis rate, which may not correlate with the total amount of protein. Therefore, we decided to extend our observations. To estimate the proportion of either protein associated in the complex under steady state conditions and to determine the molecular weight of the complex, extracts were made from nonlabeled cells. The proteins were separated by gel filtration, and fractions were analyzed by protein gel blotting using antibodies raised against both BiP and calreticulin.

Figure 7A clearly shows that BiP is present in four distinct peaks corresponding to estimated molecular masses of >400 (P1), 300 (P2), 140 (P3), and 70 kD (P4). In each of these peaks, calreticulin was seen to cofractionate with BiP, although in peaks 1 and 2 the calreticulin signal was low and easily detectable only after longer exposures (data not shown). Most of the cellular calreticulin was found in a very broad low molecular weight peak covering P3 and P4. Thus, it appears that only a very small portion of the cellular calreticulin is involved in the high molecular weight complexes (P1 and P2). However, up to 50% of BiP is present in these high molecular weight peaks. A direct comparison of the BiP signals with the calreticulin signals in each lane cannot be made because different antisera were used. The experiment does not provide insight into the number of molecules involved, but it shows the relative proportion of the molecules in distinct peaks of different molecular weights.

To determine whether BiP and calreticulin were directly associated with each other in the various peaks, the relevant fractions (indicated in Figure 7A) were pooled and immunoprecipitated using the anti-calreticulin antiserum. The pellets obtained were then washed in the presence or absence of ATP, and the supernatants were analyzed by protein gel blotting using anti-BiP antibodies. Figure 7B clearly shows that in the first three peaks, BiP is associated in an ATP-sensitive fashion with calreticulin. As expected, BiP was not coimmunoprecipitated with calreticulin in the sample from peak 4, which corresponds to a molecular weight lower than the sum of BiP and calreticulin and therefore contains unassociated monomers of the two proteins. Figure 7B also shows that nearly all of the BiP molecules present in P1, P2, and P3 are associated with calreticulin and can be released by ATP (see legend to Figure 7B for loading details). Because this represents >50% of the total BiP, the results suggest that most of the cellular BiP is trapped in complexes containing a very small proportion of the total cellular calreticulin.

DISCUSSION

BiP and Calreticulin Form an Abundant Complex

We have clearly established that the ER resident proteins BiP and calreticulin are present in an abundant complex both in the presence and absence of stress and that BiP is the most abundant protein associated with calreticulin. This
observation alone does not establish which molecule is binding and which is the ligand. Calreticulin has a clearly established lectin binding activity (Spiro et al., 1996), but BiP is not a glycoprotein. According to our current understanding of calreticulin function, it is unlikely that calreticulin would bind to BiP. The fact that the complex can be dissociated by ATP suggests that BiP is the binding protein and calreticulin the ligand; however, this observation alone is insufficient to identify the ligand and the binding protein. BiP usually binds only to intermediates of folding and assembly and to permanently defective proteins. Because calreticulin is a very abundant protein in the plant ER lumen, there could indeed be sufficient folding intermediates presenting themselves as substrates for BiP. However, we have obtained several lines of evidence contradicting such a possibility.

(1) The complex is abundant in the absence of stress. The presence of stress should promote malfolding of calreticulin and lead to an increase in BiP binding. This is not the case.

(2) During heat shock, BiP binds to unlabeled calreticulin present before pulse labeling, showing that BiP interacts with calreticulin that has been given sufficient time to fold. (3) The interaction of BiP and calreticulin is stable, with no significant dissociation or degradation of one of the partners being observed after 8 hr of chase. Unless calreticulin requires >8 hr to fold correctly, these results support our model of BiP-associated calreticulin being correctly folded. (4) Calreticulin masks BiP epitopes that are not masked by unassembled phaseolin (a model ligand for BiP), suggesting that it interacts with a different portion of the molecule. One model of BiP action is to retain improperly folded or unassembled proteins in the ER via its ER retention motif. Phaseolin does not mask the C terminus and keeps the ER retention motif accessible. However, as indicated in Figure 8, calreticulin does mask the C terminus of BiP, which represents a completely different type of interaction. (5) Overproduction of calreticulin does not lead to titration of free BiP. If a portion of calreticulin is not folded, overproduction should lead to further accumulation of unfolded calreticulin. The results show that overproduction of calreticulin leads to an increase in free calreticulin and not to additional complex formation. In contrast, BiP overproducers seem to have more BiP.

Figure 7. BiP and Calreticulin Form Large Protein Complexes.

(A) Five hundred microliters of a 2.5 mg/mL extract of suspension-cultured tobacco cells was separated on a Superose 12 column using a Pharmacia fast-protein liquid chromatography system. The resulting fractions were then analyzed by protein gel blotting, using both anti-BiP and anti-calreticulin antisera. The positions of BiP and calreticulin (Cal) are indicated at left. The estimated molecular masses of the complexes formed by BiP and calreticulin are >400 (P1), 300 (P2), 140 (P3), and 70 kD (P4). The latter corresponds well with the monomeric forms of BiP and calreticulin.

(B) The indicated peak fractions from (A) were individually pooled, and 50 µL of pools P1 and P2 were immunoprecipitated with anti-calreticulin antibodies and 10 µL of protein A-Sepharose gel. To avoid saturation of the anti-calreticulin antiserum, only 10 µL was used for P3 and P4. The pellets obtained were then washed in 30 µL of BiP release buffer in the presence (+) or absence (−) of 3 mM ATP to release coprecipitated material. Twenty microliters of clear supernatant was then analyzed by protein gel blotting using anti-BiP antibodies representing 50% of the total material. Ten microliters of protein A-Sepharose gel and 10 µL of supernatant were not added to avoid contamination by antibodies. The control lane (C) was loaded with 20 µL of starting material (fraction P1) for comparative purposes. Note that the intensity of the BiP bands in P1 and P2 is comparable to that of the control lane, which would be expected if most of the BiP in these fractions is associated with calreticulin. The intensity of the BiP band in P3 is lower, but this is because five times less material was used for the immunoprecipitation (see above). The position of BiP is indicated at left.

Figure 8. Model for BiP-Calreticulin Interaction.

A schematic representation of the interaction of BiP with assembly-defective phaseolin (Phas) and with calreticulin (Calret). The antibody binding sites of BiP remain exposed when bound to phaseolin but are masked by the interaction with calreticulin. This indicates that a different portion of the BiP molecule is involved in the interaction.
recruited into complexes with calreticulin, as shown by a higher proportion of coprecipitating BiP compared with precipitated calreticulin.

Together, the data clearly suggest that BiP is not bound to unfolded or malfolded calreticulin and that the complex must have another function. Our results extend recent data obtained from cross-linking experiments, which revealed interactions between several ER chaperones, including BiP and calreticulin (Tatu and Helenius, 1997). Consistent with our observations, such interactions were also detected when protein synthesis was inhibited, suggesting that they are not directly linked to the action on newly synthesized polypeptides in the process of folding or assembly.

**Biological Relevance of the BiP-Calreticulin Complex**

To find the biological function of the observed complex, it would be necessary to purify the complexes from the monomeric forms and to conduct experiments in vitro. We have identified, by gel filtration analysis, three distinct complexes with different molecular masses. The two high molecular mass peaks (400 and 300 kD) contain mainly BiP associated with calreticulin. An additional peak of 140 kD was found that corresponds approximately to the sum of the two molecular masses. More than 50% of the total cellular BiP is found in these complexes, whereas the vast majority of cellular calreticulin is monomeric or present in the low molecular mass peak of 140 kD. At this peak, the ratio of calreticulin to BiP is increased relative to the two other peaks. We have observed previously (Denecke et al., 1995) that a portion of calreticulin can be purified as a homomultimer because it elutes from gel filtration columns before endoplasmic reticulum. This phenomenon is currently being investigated.

Together, these observations strongly suggest that the BiP–calreticulin interaction occurs in vivo and that it is not an extraction artifact. Instead, extraction may even disrupt the complex to some extent, and it is very likely that in vivo, most of the cellular BiP is associated with calreticulin.

The results from the gel filtration experiments showed that the majority of the cellular BiP is associated with a relatively small portion of the cellular calreticulin. The excess of calreticulin also explains why calreticulin overproduction has no effect on the recruitment of BiP, whereas BiP overexpression increases the BiP/calreticulin ratio in the complexes. In addition, more BiP appears to be complexed with calreticulin than with an assembly-defective protein overproduced in plants. This is remarkable because the latter represents an artificial situation, and it is unlikely that under normal culture conditions, similar amounts of unfolded or malfolded proteins would be found in the ER lumen. Therefore, in the absence of stress, calreticulin is the major BiP ligand, and BiP is the major calreticulin ligand.

The fact that truncated BiP lacking the retention motif still binds to calreticulin suggests that the retention signal itself is not required for the interaction. In addition, binding of the truncated protein to calreticulin may explain why HDEL/KDEL-less BiP is not efficiently secreted in mammalian cells (Munro and Pelham, 1987) and is retained in the ER of plants almost as efficiently as is the wild-type protein (N. Leborgne-Castel and J. Denecke, manuscript in preparation). This will provide an interesting system with which to test ER retention and complex formation simultaneously with additional deletion mutants of BiP. Interestingly, overexpression of BiP appears to have an effect on the glycosylation status of calreticulin. This phenomenon is currently being investigated.

**Possible Functions of the BiP-Calreticulin Complex**

What then is the function of this complex? A role for calreticulin as a cofactor of BiP is unlikely because the BiP–phaseolin complex does not contain calreticulin. One possibility could be to provide a buffer for the concentration of free BiP. The excess of the cellular BiP would then be stored as a complex with calreticulin. Overproduction of BiP may thus lead to further recruitment of BiP into complexes with calreticulin, because the majority of BiP would not be needed for normal ER function.

The complex may also be part of some larger structure involving other ER chaperones. Such structures have been detected using cross-linking experiments (Tatu and Helenius, 1997) and could be represented by the oligomers with molecular masses of ~300 kD and >400 kD that we have been able to identify in the absence of cross-linkers. Perhaps participating proteins other than BiP and calreticulin are disrupted by our immunoprecipitation procedures but remain associated during gel filtration. Our experiments show that in the case of BiP and calreticulin, the affinity for calcium and the simple formation of calcium bridges cannot explain
of the BIP–calreticulin complex. The presence of distinct oligomers suggests that the interactions holding together the putative chaperone matrix in the ER (Hammond and Helenius, 1995) would be diverse and partner specific. Clearly, it is important to conduct further research to elucidate the function of this novel complex in the ER lumen. The purification of the complex would help to determine its composition and is certainly an immediate priority.

METHODS

Protoplast Preparation and Labeling

Tobacco (Nicotiana tabacum) leaf protoplasts were produced as described by Denecke and Vitale (1995) and incubated at a cell density of 10^6 protoplasts per mL in 15-mL Falcon tubes (Becton Dickinson & Co., Lincoln Park, NJ) (1 mL per tube). Protoplasts were incubated at room temperature or at 37°C during heat shock treatment for 3 hr in the presence of 100 μCi/mL Pro-mix (Amersham Life Science; containing 70% 35S-methionine and 30% 35S-cysteine). Pulse–chase experiments used a 1-hr labeling and chase buffer containing 10 mM methionine and 5 mM cysteine. All other manipulations were done essentially as described by Denecke and Vitale (1995).

Protoplast Homogenization

Protoplasts were resuspended and lysed in 4 volumes of freshly prepared ice-cold homogenization buffer (200 mM Tris-Cl, pH 8.0, 300 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride). The homogenate was then centrifuged for 5 min in a minicentrifuge, and the supernatant was aliquoted and stored at −80°C.

Immunoprecipitation

All manipulations were performed on ice or at 4°C using ice-cold buffers. A fraction of the homogenate was brought to room temperature or at 37°C using ice-cold water. A fraction of the homogenate was brought to a final activity is 20 milliunits per mL. For the control digest, 10 μL of citrate buffer alone was added to the protein A pellet. Both digest and control reactions were then incubated at 37°C for 2 hr. Twenty microliters of SDS sample buffer was added, and boiling, centrifugation, and loading for SDS-PAGE were performed as described in the previous paragraph.

Endoglycosidase H Digestion

Ten microliters of endoglycosidase H solubilized in 100 mM sodium citrate, pH 5.5 (40 milliunits per mL), was added to the first protein A-Sepharose pellet. Because the volume of the pellet is ~10 μL, the final activity is 20 milliunits per mL. For the control digest, 10 μL of citrate buffer alone was added to the protein A pellet. Both digest and control reactions were then incubated at 37°C for 2 hr. Twenty microliters of SDS sample buffer was added, and boiling, centrifugation, and loading for SDS-PAGE were performed as described in the previous paragraph.

Gel Filtration

Extracts for gel filtration experiments were prepared from suspension-cultured BY2 AP tobacco cells by using a prechilled Waring blender and ice-cold extraction buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂. After extraction, the cell homogenate was centrifuged at 4°C to remove cell debris, aliquoted, and stored at −20°C. Gel filtration was performed using a Pharmacia fast-protein liquid chromatography machine and a Superose 12 column, which had previously been calibrated with a range of protein standards to allow molecular weight determination.

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