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A soybean binding protein (BiP) homolog is temporally regulated in soybean seeds and associates detectably with normal storage proteins *in vitro*

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ABSTRACT

The endoplasmic reticulum (ER) luminal binding protein (BiP) is thought to be a key mediator of folding and assembly of *de novo* synthesized secretory proteins. We have used a maize (*Zea mays* L.) BiP antibody to identify its homolog in soybeans (*Glycine max* (L.) Merril). The accumulation of BiP in developing soybean seeds seems to be coordinated with the onset of active storage protein synthesis. We used a co-immunoprecipitation assay to detect soybean BiP: β-conglycinin interactions. Either a maize BiP antibody or a β-conglycinin antibody co-immunoprecipitated the reciprocal protein from whole seed protein extract enzymatically depleted of adenosine 5'-triphosphate (ATP), while an unrelated antibody failed to immunoprecipitate either one. The association of BiP:β-conglycinin complexes was completely reversed by addition of ATP, a diagnostic feature of molecular chaperone-mediated interaction. However, only a very small fraction of β-conglycinin was found to be associated with BiP in whole cell protein extracts from immature seeds. These results are consistent with a transient association between BiP and β-conglycinin subunits, and suggests its involvement in the biosynthetic transport pathway of storage proteins to protein bodies.

INTRODUCTION

The heat shock proteins (HSP70) are a ubiquitous family of stress-induced proteins, which have been described as polypeptide binding proteins and molecular chaperones (revised in Hendrick and Hartl, 1993). In eukaryote, the HSP70-related proteins belong to a multigene family whose members are targeted to different sub-cellular compartments (for review, see Ellis, 1991). Among members of the HSP70 family, structural differences, related to targeting signals, have been used to assign the conserved members of this family to its sub-cellular localization. The endoplasmic reticulum (ER)-resident HSP70 protein, known as BiP (binding protein), has a functional peptide signal at the amino terminus which directs it synthesis to the ER and a tetrapeptide carboxyl-terminal, K/R/HDEL, which constitutes a general reticuloplasmin retention signal (Munro and Pelham, 1987; Pelham, 1989; Denecke *et al.*, 1992). The retrieval of BiP in the ER is mediated by the interaction with the HDEL/KDEL receptor protein (Pelham, 1988; Semenza *et al.*, 1990; Lee *et al.*, 1993).

As a member of the HSP70 family, the molecular chaperone activity of BiP has been characterized in several systems (for review, see Vitale *et al.*, 1993). BiP

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has been described as an important component of a quality control system in the ER, in which a sorting mechanism of protein exiting from the organelle is mediated by protein:protein interaction. BiP associates transiently with nascent polypeptides to promote proper folding and assembly of the associated proteins, and to prevent aggregations of folding intermediates in the ER (Hendershot, 1990; Roux, 1990; Knittler and Haas, 1992). Stable binding of BiP reflects failure of the associated proteins to achieve a proper conformation, which prevents secretion of misfolded or incompletely assembled proteins (Bole *et al.*, 1986; Gething *et al.*, 1986; Kassenbrock *et al.*, 1988; Hurtley *et al.*, 1989). BiP is also involved in the translocation pathway of newly synthesized polypeptide to the lumen of the ER (Vogel *et al.*, 1990; Nguyen *et al.*, 1991). The BiP-mediated translocation of nascent polypeptides is considered to be driven by its protein binding activity which prevents proteins aggregation that would impair translocation through the ER membranes.

The evolutionary conservation of BiP has been examined in several eukaryotic systems. Sequence comparison analyses of cDNA clones isolated from mammalian cells have demonstrated a high degree of conservation at the amino acid sequence level (Chang *et al.*, 1987; Haas and Meo, 1988; Ting and Lee, 1988). Conservation of primary structure extends to include yeast and plant counterparts (Normington *et al.*, 1989; Rose *et al.*, 1989; Denecke *et al.*, 1991; Fontes *et al.*, 1991; Anderson *et al.*, 1993). In addition to the HDEL/KDEL carboxyl-terminal tetrapeptide sequence shared by all BiP homologs so far examined, they have an ATP-binding site, and exhibit ATPase activity (Munro and Pelham, 1986; Kassenbrock and Kelly, 1989). Binding of ATP to BiP prevents formation of BiP:protein complexes and hybrolysis of ATP promotes BiP dissociation (Munro and Pelham, 1986; Hurtley *et al.*, 1989).

BiP is expressed at a low level in normal growth environments. Induction of BiP synthesis is triggered by treatment of the cells with different physiological stresses. These include glucose starvation, treatment with inhibitors of protein glycosylation, such as tunicamycin and 2-deoxy glucose, treatment with 2-mercaptoethanol, amino acid analogs, and the calcium ionophore (Lee, 1987). These BiP inducers are dissimilar and result in the accumulation of misfolded proteins in the ER. Furthermore, the synthesis of aberrant secretory proteins causes BiP induction in untreated cells (Kassenbrock *et al.*, 1988). The induction of BiP under conditions that promote protein denaturation, associated with its protein binding activity, supports the argument that during stress BiP binds to abnormal or underglycosylated proteins and prevents their aggregation (Bole *et al.*, 1989; Hendershot, 1990).

Several cDNAs encoding BiP, which show functional conservation with their animal and yeast homologs, have been isolated from plants. The protein binding activity of plant BiP has been examined under stress and normal conditions. Maize, tobacco (*Nicotiana tabacum* L.), and bean (*Phaseolus vulgaris* L.) BiP homologs have been shown to be induced upon treatment of plant cells with tunicamycin (Denecke *et al.*, 1991; Fontes *et al.*, 1991; D'Amico *et al.*, 1992). Underglycosylated phaseolins from beans and an assembly-defective phaseolin mutant have been demonstrated to associate with BiP (D'Amico *et al.*, 1992; Pedrazzini *et al.*, 1994). BiP is overexpressed in endosperm mutants of maize which accumula abnormally shaped protein bodies (Boston *et al.*, 1991; Fontes *et al.*, 1991; Zhang and Boston, 1992). These morphological abnormalities are thought to be a result of storage proteins incorrectly assembled during biogenesis of protein bodies. BiP has also been shown to associate *in vitro* with newly synthesized prolamins from rice (*Oryza sativa* L.) to facilitate their folding and assembly into protein bodies that emerge from the ER (Li *et al.*, 1993).

Unlike rice, the major pathway of protein body formation in developing soybean cotyledons involves a Golgi-mediated transport of storage proteins to the vacuoles where they accumulate into protein bodies (Chrispeels, 1984). The major storage proteins from soybean, glycinin and β-conglycinin, are synthesized on membrane-bound polysomes as precursors containing functional signal peptides. These N-terminal sequences direct import into the rough endoplasmic reticulum (RER), where processed glycinin and β-conglycinin are assembled into trimers. Subsequently, the oligomeric proteins are transported via Golgi complex to the vacuolar protein bodies. Protein translocation and folding in the lumen of ER is one of the limiting steps for intracellular protein transport in the secretory pathway.

MATERIAL AND METHODS

Plant material

All soybean varieties were grown under standard greenhouse conditions. For protein isolation, seeds were harvested at the developmental stages indicated, immediately frozen in liquid nitrogen and stored at -80°C.

Purification of monospecific anti-soybean BiP antibodies

Bacteriophage λgt11 harboring a 2.4-kb cDNA insert for soybean BiP (to be described elsewhere) was grown on *E. coli* strain LE 392 and immobilized on nitrocellulose filters. The filters were subsequently incubated with polyclonal maize BiP antiserum (Fontes *et al.*, 1991) to affinity-purify antibodies specific for β-galactosidase soybean-BiP fusion protein. The monospecific antibodies were eluted with 0.1 M glycine, pH 2.9, and immediately neutralized (Choi *et al.*, 1987). The eluted antibody, referred to as clone-selected antibody, was stored in 10 mM Tris-HCl, pH 7.4 at 25°C, 0.14 M NaCl and 0.2% (w/v) NaN_3.

Protein extraction and immunoblot analysis

For protein extraction, all the steps were performed at 4°C. Whole cell protein extracts were prepared from soybean seeds at the developmental storage indicated. Frozen seeds were ground with a pestle and a mortar with 50 mM Tris-HCl, pH 7.5 at 25°C, 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma). Cell debris was removed by centrifugation at 16,000 *g* for 15 min. For the developmental series, protein from an equal fresh weight of seeds was electrophoresed on 10% SDS polyacrylamide gel (SDS-PAGE), transferred to nitrocellulose and visualized using a rabbit polyclonal antibody to maize BiP or anti-soybean BiP clone selected antibody and a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). Alkaline phosphatase activity was detected with BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) and NBT (nitroblue tetrazolium) substrates (GIBCO BRL). For seedlings and leaves, the extraction buffer was 50 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5 at 25°C, 1% Triton X-100, 1 mM PMSF and 100 µg of total protein was electrophoresed and electroblotted.

Co-immunoprecipitation assay

For the immunoprecipitation assays, rabbit anti-maize BiP antiserum was cross-linked to Protein-A Sepharose beads (Sigma) as described by Simanis and Lane (1985). A chicken anti-β-conglycinin antiserum was linked to CNBr-activated Sepharose beads (Pharmacia) according to manufacturer's instructions. All the immunoprecipitation steps were performed at 4°C. Protein extracts were prepared by homogenization of immature seeds with 50 mM Tris-HCl, pH 7.5 at 25°C, 100 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM PMSF, 2 U/ml hexokinase (MERCK) and 5 mM glucose at a ratio of 1 g of the tissue/5 ml of extraction buffer. After incubation of the protein extract for 10 min, cell debris was removed by centrifugation at 13,000 *g* for 15 min. The supernatant (50-100 mg) was precleared with 0.1 ml of 50% (v/v) Protein A-Sepharose suspension in TBS (20 mM Tris-HCl, pH 7.5 at 25°C and 140 mM NaCl) and immunoprecipitated overnight with 0.1 ml of the appropriate polyclonal antiserum previously linked to Sepharose beads (50% slurry in TBS). The immunocomplexes were recovered by centrifugation at 8,000 *g* for 5 min. Pelleted Sepharose beads were washed extensively with 1 ml of 50 mM Tris-HCl, pH 7.5 at 25°C, 0.5 M NaCl, 1% (v/v) Triton X-100, 1 mM PMSF and resuspended in 40 µl of SDS-PAGE sample buffer (Laemmli, 1970). Immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblot analysis using either clone-selected anti-BiP antiserum and a goat anti-rabbit IgG alkaline phosphatase-conjugate (Sigma) or a chicken anti-β-conglycinin antiserum and a rabbit anti-chicken IgG alkaline phosphatase-conjugate (Sigma).

RESULTS AND DISCUSSION

Specificity of clone selected antibody

To identify a soybean BiP homolog we used an antibody raised against a purified maize BiP (Fontes *et al.*, 1991). A 72-75-kDa protein, immunologically related and identical in relative molecular mass (Mr) to the maize BiP, was detected in ER fractions of soybean seeds (Figure 1, lane ER). Based on the sub-cellular localization of the anti-BiP cross-reactive protein and its electrophoretic mobility we inferred that the maize antibody recognizes a BiP homolog in soybean seeds. A polypeptide, similar in size to the anti-BiP cross-reactive protein from seeds (Figure 1, lanes ER and Se), was also detected in the whole protein extracts from leaves and seedlings (Figure 1, lanes Lf and Sd). However, several minor polypeptides cross-reacted with the anti-maize BiP antisera (Figure 1). Because the polyclonal maize BiP antisera cross-reacted with several proteins, we used an immunoaffinity fractionation of the maize BiP polyclonal antibody to select soybean BiP epitopes. The rabbit anti-maize BiP antiserum was affinity-purified by selection with a β-galactosidase soybean BiP fusion protein (see Methods). This clone-selected antibody recognizes only the soybean BiP homolog in whole cell protein extracts from seeds, leaves and seedlings (Figure 2 and data not shown).

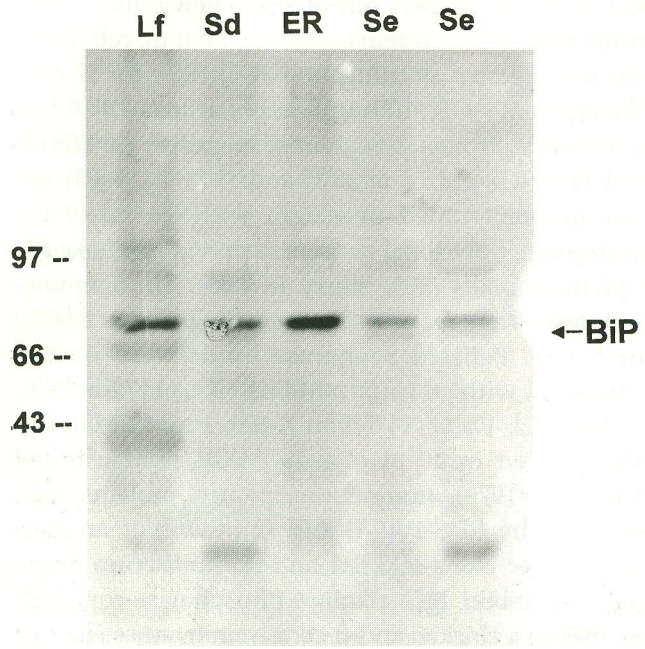

Figure 1 - Immunoblot of soybean BiP homolog. Proteins (50 µg) from whole cell extracts of leaves (Lf), seedling (Sd) and seeds (lanes Se) were electrophoresed and immunoblotted using an anti-maize BiP antiserum. Lane ER corresponds to the ER fraction isolated from seeds. BiP is indicated on the right. The sizes and positions of protein molecular mass markers are shown on the left in kDa.

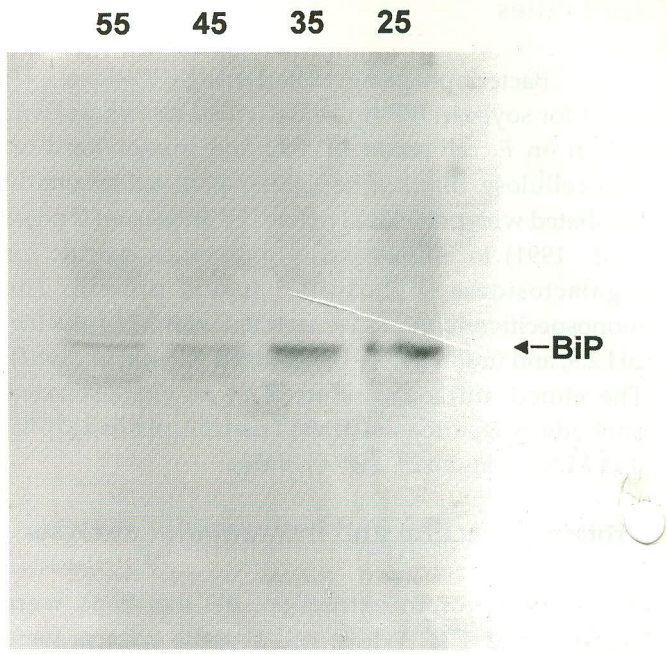

Figure 2 - Developmental accumulation of BiP in soybean seeds. Soluble protein extracts of soybean seeds were resolved in SDS-PAGE and immunoblotted using soybean clone selected antibody. Protein from equal fresh weights of seeds was loaded in each lane. Developmental stages are indicated on the top in days after flowering (DAF). The position of BiP is shown on the right.

Developmental accumulation of BiP in soybean seeds

The recent identification of enhanced synthesis of BiP in secretory tissues of plants prompted us to investigate the requirement of BiP synthesis during development of the seed. As shown in Figure 2, BiP is detectable from 25 days after flowering (DAF) to seed maturation, but it is most abundant during early stages of cotyledon development. The rate of BiP synthesis seems to be coordinated with active synthesis of storage proteins. At 25 to 35 DAF, storage protein gene transcription is maximized and presumably so is translocation of proteins through the ER membranes (Walling *et al.*, 1986). These results may reflect a stimulation of BiP accumulation in soybean seeds as a result of increased synthesis of newly synthesized storage proteins.

Transient association between BiP and β-conglycinin subunits

Because the expression of the soybean BiP homolog seems to be coordinated with active synthesis of storage proteins, we used a co-immunoprecipitation assay to determine the ability of BiP to interact with subunits of β-conglycinin. For the detection of BiP:β-conglycinin association, we used extracts from a stage of cotyledon (25 DAF) where BiP accumulation and β-conglycinin gene transcription are maximized, but large amounts of storage protein are not accumulated in protein bodies (Walling *et al.*, 1986). Immunoblots of whole cell protein extracts showed that antibodies to BiP and to β-conglycinin subunits do not cross-react with either of the reciprocal proteins (Figure 3, lane 6 and data not shown). BiP was co-immunoprecipitated, however, by antibodies against β-conglycinin in the absence (lane 4) but not in the presence of ATP (lane 5). The precipitation of BiP by anti-β-conglycinin antisera was not due to non-specific binding, because antibodies to an unrelated protein failed to precipitate BiP (lane 2). Because ATP did not prevent direct immunoprecipitation of BiP by anti-maize BiP antisera (lanes 1 and 3), we concluded that the BiP:β-conglycinin complexes detected *in vitro* are sensitive to ATP, a biochemical property of molecular chaperone-mediated interactions.

Likewise, in the reciprocal experiment, the β-conglycinin subunits, α' and α, were immuno-

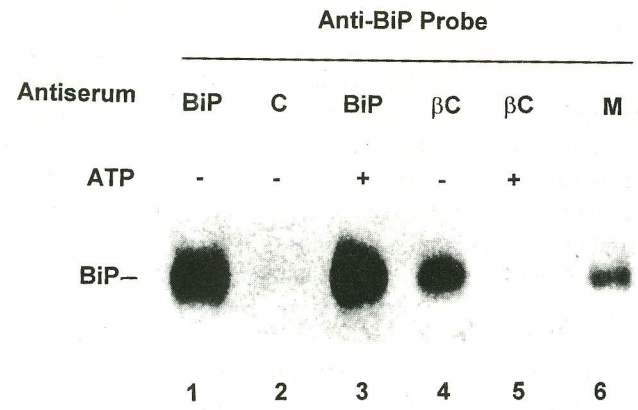

Figure 3 - Immunoprecipitation of BiP and soybean storage proteins. Immunoprecipitation reactions were carried out using rabbit anti-maize BiP antiserum cross-linked to Protein-A Sepharose (BiP), chicken anti-β-conglycinin antiserum linked to CNBr-Sepharose 4B (βC) or control antiserum cross-linked to Protein-A-Sepharose (C). BiP and β-conglycinin subunits were immunoprecipitated from 1 ml of whole cell protein extracts prepared from soybean seeds of the variety UFV-5 harvested 25 days after flowering. Immunoprecipitated products were immunoblotted and probed with clone-selected anti-BiP antiserum. Initial immunoprecipitations were performed with anti-BiP antiserum minus ATP (lane 1); unrelated antiserum minus ATP (lane 2); anti-BiP antiserum plus ATP (lane 3); anti-β-conglycinin antiserum minus ATP (lane 4); anti-β-conglycinin plus ATP (lane 5). Lane 6 contains 50 µl of the initial protein extract. The position of BiP is shown on the left of the blot.

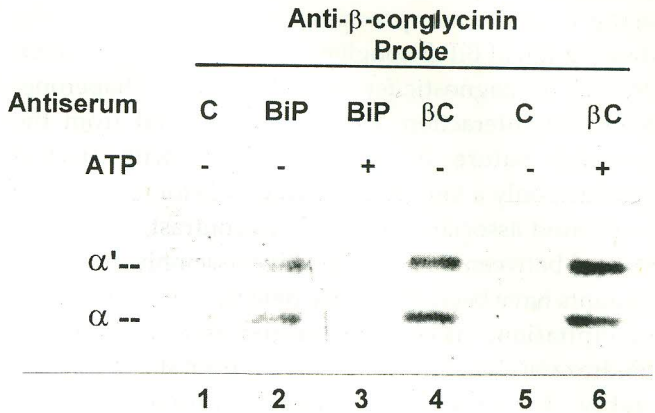

Figure 4 - Immunoblot of immunoprecipitated products probed with anti-β-conglycinin antiserum. Immunoprecipitation reactions were carried out as described in Figure 3, except that immunoblots were probed with a chicken anti-β-conglycinin antiserum and rabbit anti-chicken IgG secondary antibody conjugated to alkaline phosphatase. Immunoprecipitations were performed with control antibody minus ATP and 500 µl extract (lane 1), anti-BiP antiserum minus ATP and 500 µl extract (lane 2), anti-BiP antiserum plus ATP and 500 µl extract (lane 3), anti-β-conglycinin antiserum minus ATP and 0.5 µl extract (lane 4), unrelated antiserum and 0.5 µl extract (lane 5), anti-β-conglycinin antiserum plus ATP and 0.5 µl extract (lane 6). The positions of α' and α subunits are shown on the left of the blot.

precipitated by antibodies to BiP (Figure 4, lane 2) and to β-conglycinin (lanes 4 and 6), but not by antibodies against an unrelated control protein (lanes 1 and 5). BiP:β-conglycinin subunits were formed only in the ATP-depleted condition (lane 2). Inclusion of ATP in the immunoprecipitation assay prevented BiP:β-conglycinin association (lane 3). Direct immunoprecipitation of α' and α subunits from 0.5 µl extract was as efficient as indirect immunoprecipitation of the same polypeptides from 500 µl extract using anti-BiP antiserum (Figure 4, compare lanes 2 and 4). The concentration of free β-conglycinin was at least 1000-fold higher than BiP-associated β-conglycinin. This ratio is not surprising since the interaction of BiP with normal proteins is expected to be very transient whereas interaction with assembly-deficient phaseolin mutants has been observed to be much greater (Pedrazzini *et al.*, 1994). Thus, the efficiency of BiP dissociation from β-conglycinin subunits and the subsequent assembly of the released subunits in their trimeric forms may be very high.

The storage proteins in legume seeds are synthesized during seed development and are accumulated in small membrane-bounded protein bodies within the cotyledon cells. To obtain insights into the molecular mechanisms involved in the intracellular transport of storage proteins to the vacuoles, we identified and characterized a BiP homolog in soybean seeds. Analyses of the pattern of BiP accumulation in soybean seeds demonstrate that BiP is more abundant at early stages of cotyledon development (Figure 2). Accumulation of BiP transcripts is coordinated with that of BiP protein during seed development (data not shown). The temporal regulation of BiP synthesis in soybean seeds may be a secondary effect on BiP gene expression because of increased secretory activity associated with enhanced synthesis of storage proteins within the cotyledon. In mammalian cells, the accumulation of secretory precursors in the lumen of ER causes an increase in the rate of BiP synthesis (Normington *et al.*, 1989; Rose *et al.*, 1989). Tobacco BiP mRNA transcripts also accumulate predominantly in secretory tissue and in tissue with a high rate of cell division (Denecke *et al.*, 1991). These results suggest that the rate of BiP synthesis in unstressed cells depends on the secretory activity of the cell. A corollary of this hypothesis is that BiP accumulates predominantly in cotyledon cells during active storage protein synthesis.

Evidence suggests that the soybean BiP homolog is functionally analogous to molecular chaperones. First, we detected BiP:storage protein

interaction in protein extracts from immature seeds using a co-immunoprecipitation assay. Second, the dissociation of BiP:β-conglycinin complex is dependent on ATP, a diagnostic feature of molecular chaperone-mediated interaction. Finally, as expected from the transient nature of BiP association with normal proteins, only a very small fraction of storage proteins was found associated with BiP. In contrast, complexes formed between BiP and defective-assembly phaseolin mutants have been efficiently detected by co-immunoprecipitation assay in protoplasts of tobacco (Pedrazzini *et al.*, 1994). BiP has also been shown to bind stably to underglycosylated storage proteins synthesized in tunicamycin-treated bean cotyledon (D'Amico *et al.*, 1992). Stable binding of BiP to secretory proteins in the lumen of the ER reflects failure of the proteins to achieve a proper conformation, a pre-requisite for subsequent trafficking through the secretory pathway (Bole *et al.*, 1986; Gething *et al.*, 1986; Kassenbrock *et al.*, 1988; Hurtley *et al.*, 1989). The association of BiP with normal polypeptide involves transient binding because BiP is released when proper folding or assembly is achieved (Hendershot, 1990; Roux, 1990; Knittler and Haas, 1992). The low detectable level of BiP-associated storage protein in our assay suggests that the efficiency of BiP dissociation from β-conglycinin subunits and the subsequent assembly of the subunits in their trimeric form is very high.

Our results are consistent with the involvement of BiP in the assembly and transport of normal storage proteins to the protein bodies. In this case, one may expect that BiP participates actively in the process of folding and assembly of storage proteins in the lumen of the ER. *In vitro* synthesized glycinin and β-conglycinin polypeptides retain the ability to self-assemble properly into trimers in a cell-free system (Dickinson *et al.*, 1987). Although BiP levels were not analyzed in the transcription/translation system, most likely the presence of functional BiP homologs in rabbit reticulocyte lysates provided the molecular chaperone activity required for assembly.

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RESUMO

A proteína BiP, residente no lúmen do retículo endoplasmático, tem sido descrita como um importante componente do processo de dobramento e montagem de proteínas secretórias recém-sintetizadas. Com a finalidade de se identificar a proteína homóloga de BiP da soja, anticorpos contra BiP do milho foram usados como sonda em "westerns" de extratos de proteínas da semente, folha e plântula. O acúmulo de BiP em sementes em desenvolvimento parece estar coordenado com a atividade de síntese de proteínas de reserva. Um ensaio de co-imunoprecipitação foi usado a fim de se determinar se BiP interage com proteínas de reserva. Na ausência de ATP, anticorpos contra BiP do milho ou contra β-conglicinina imunoprecipitaram a proteína recíproca, enquanto que anticorpos contra uma proteína não relacionada não a precipitaram. Na presença de ATP, complexo formado entre BiP e β-conglicinina não pode ser detectado. A dissociação de interações protéicas pela hidrólise de ATP constitue um diagnóstico bioquímico de interações mediadas por chaperones moleculares. No entanto, em extratos de proteína total de sementes imaturas, apenas uma pequena fração de β-conglicinina pode ser detectada em associação com BiP. Conseqüentemente, pode-se antecipar que a associação do complexo BiP:β-conglicinina e subseqüente montagem do complexo trimérico das subunidades de β-conglicinina deve ocorrer com alta eficiência. Estes resultados são consistentes com o envolvimento de BiP no transporte biossintético de proteínas de reserva para os corpos protéicos.

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