Characterization of an Immunoglobulin Binding Protein Homolog in the Maize *floury-2* Endosperm Mutant

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The maize *b-70* protein is an endoplasmic reticulum protein overproduced in the *floury-2* (*f12*) endosperm mutant. The increase in *b-70* levels in *f12* plants occurs during seed maturation and is endosperm specific. We have used amino acid sequence homology to identify *b-70* as a homolog of mammalian immunoglobulin binding protein (*BiP*). Purified *b-70* fractions contain two 75-kilodalton polypeptides with pl values of 5.3 and 5.4. Both 75-kilodalton polypeptides share several properties with *BiP*, including the ability to bind ATP and localization within the lumen of the endoplasmic reticulum. In addition, both *b-70* polypeptides can be induced in maize cell cultures with tunicamycin treatment. Like *BiP*, the pl 5.3 form of *b-70* is post-translationally modified by phosphorylation and ADP-ribosylation. However, modification of the pl 5.4 species was not detected in vitro or in vivo. Although the *b-70* gene is unlinked to *f12*, *b-70* overproduction is positively correlated with the *f12* gene and is regulated at the mRNA level. In contrast, the *f12* allele negatively affects the accumulation of the major endosperm storage proteins. The physical similarity of *b-70* to *BiP* and its association with abnormal protein accumulation in *f12* endoplasmic reticulum may reflect a biological function to mediate protein folding and assembly in maize endosperm.

INTRODUCTION

Biosynthetic protein transport in eukaryotic cells involves import of newly synthesized polypeptides into the lumen of endoplasmic reticulum (ER). Once there, the polypeptides may achieve a translocation-competent conformation leading to assembly into protein oligomers, transport to other subcellular organelles, or secretion from the cell (Pfeffer and Rothman, 1987). Recent studies have indicated that proper protein conformation is required for transport from the ER (for review, see Rose and Doms, 1988). ER-resident proteins may facilitate this process by catalyzing the correct folding and assembly of polypeptides into oligomers.

An ER-localized protein that has been shown to have such polypeptide chain-binding properties is the immunoglobulin binding protein (*BiP*) (Haas and Wabl, 1983). A member of the heat shock 70-related protein (*hsp70*) family, *BiP* binds transiently to newly synthesized proteins or permanently to incorrectly folded proteins in mammalian cell culture systems (Gething et al., 1986; Kassenbrock et al., 1988). In addition, *BiP* has been implicated as a catalyst of protein folding and assembly (Bole et al., 1986; Gething et al., 1986; Hurtley et al., 1989; Ng et al., 1989; Roux, 1990). *BiP* accumulation is induced by inhibitors of protein glycosylation, calcium ionophores, and conditions that promote protein denaturation and aggregation (for review, see Lee, 1987). In addition, *BiP* binds ATP with high affinity and is post-translationally modified by phosphorylation and ADP-ribosylation (Carlsson and Lazarides, 1983; Hendershot et al., 1988; Kassenbrock and Kelly, 1989).

The accumulation of zeins, the major storage protein reserves in the endosperm of maize kernels, involves import of newly synthesized proteins into the ER. Once there, these hydrophobic, alcohol-soluble proteins are deposited into dense, membrane-bound protein bodies (Larkin and Hurkman, 1978). The mechanisms of zein targeting and assembly in protein bodies are poorly understood. Abundant α-zeins appear to accumulate and assemble into locules that coalesce into a central aggregate surrounded by a shell of β-zeins and γ-zeins (Lending et al., 1988). Structural and sequence analyses led to the theory that zein aggregation in protein bodies is driven by inter-
actions among the proteins themselves (Burr and Burr, 1976; Argos et al., 1982). Observations that zeins assembled into protein body-like organelles when they were expressed in a Xenopus oocyte system are consistent with such self-assembly (Hurkmans et al., 1981). However, data from zein assembly studies do not exclude protein-mediated packing of zeins in maize endosperm and even in the heterologous Xenopus oocyte systems.

The transport machinery of eukaryotic cells and, in some cases, the targeting signals for protein compartmentalization, are shared among diverse organisms (Pfeffer and Rothman, 1987; Gould et al., 1990). This conservation is also exhibited by the ER-resident BiP (Ting et al., 1987; C. Lending, personal communication). Although the molecular basis of the mutation is not known, BiP has been detected in association with normal and abnormal proteins in the ER and is thought to be an important component of the protein processing and secretory pathway (Hendershot, 1990).

In maize, mutation at the floury-2 (fl2) regulatory locus is associated with reduction in zein synthesis and alteration in protein body morphology (Christianson et al., 1974; Jones, 1978). Unlike normal spherical protein bodies, fl2 protein bodies are irregularly shaped and exhibit an altered arrangement of the storage proteins within them (Lending et al., 1988; C. Lending, personal communication). Although the molecular basis of the mutation is not known, fl2 kernels overproduce a soluble protein with an apparent molecular weight of approximately 70,000. Cellular fractionation and subsequent electrophoretic analysis of the protein fractions have revealed that this protein, b-70, is associated with both protein bodies and rough endoplasmic reticulum membranes (Galante et al., 1983; Salamini et al., 1985). The altered accumulation of b-70 and zein, as well as the deformation of protein bodies, reflects the semidominance of the fl2 gene (Jones, 1978; Galante et al., 1983; Boston et al., 1991).

In the accompanying paper (Boston et al., 1991), we show that b-70 is also overproduced in endosperm of two other zein regulatory mutants, Defective endosperm-B30 and Muconate. In each of these mutants, increases in b-70 protein levels reflect the onset of zein accumulation in endosperm. In addition, the changes in b-70 protein levels are regulated, in part, at the level of mRNA accumulation. Although the biological function of b-70 is not known, its subcellular localization, pattern of accumulation in fl2 genotypes, and association with abnormal protein bodies are suggestive of a biological function in the zein secretory pathway.

In this paper, we describe the purification and biochemical characterization of the maize b-70 protein. We present evidence that b-70 is a member of the hsp70-related family of proteins. Our data support the assignment of b-70 as a plant homolog of the mammalian BiP. We show that b-70 and BiP share amino acid sequences and biochemical properties, and respond similarly to specific stresses.

### Figure 1. Amino Acid Sequences of hsp70, b-70, KAR2, and BiP Proteins.

The deduced amino acid sequence from cb70-1 (b-70) is compared with the maize hsp70 (Rochester et al., 1986), mouse BiP (Kozutsumi et al., 1989), and yeast KAR2 proteins (Rose et al., 1989) according to an alignment generated by the FASTA program of the University of Wisconsin Genetics Computer Group. The amino-terminal sequence b-70 is the deduced amino acid sequence of PCR-generated b-70 clones from W64A/fl2 mRNA. Dots indicate homology to amino acids in the b-70 sequence, spaces represent gaps that were introduced when necessary to preserve alignment, numbering is relative to the b-70 sequence encoded by the cDNA clone, and asterisks represent termination codons.
Characterization of a Maize Molecular Chaperone

Figure 1 shows an amino acid comparison of b-70, maize hsp70, mouse BiP, and the yeast BiP homolog KAR2. Homology is found between b-70 and maize hsp70 at 62% of the amino acids encoded by the b-70 cDNA clone. Perhaps more striking are the comparisons of b-70 with BiP and KAR2. These alignments were also found to be identical at 62% of the residues even though the organisms represent extremes of eukaryotic divergence.

The carboxy-terminal sequence of BiP homologs is H/KDEL, an ER retention signal characteristic of many resident ER proteins (reviewed in Pelham, 1989). Although such a sequence can be deduced from the sequence of cb70-1, it precedes the carboxy terminus by 19 amino acids. The association of b-70 with ER-derived protein bodies prompted us to analyze b-70 mRNAs further for the presence of a stop codon immediately following the HDEL region. Oligonucleotide primers were synthesized to allow amplification of b-70 cDNAs corresponding to the 3’ end of mRNA from W64A/f12 kernels. Amplification products were cloned and the 3’ DNA sequences determined. From five of five subclones tested, all contained an A-to-G transition relative to the initial cDNA clone. This change

Figure 2. Purification of b-70 from 24 DAP Kernels.

The polypeptide composition of fractions from sequential steps of a b-70 preparation from f12 kernels was monitored by Coomassie staining of SDS-polyacrylamide gels as follows. Lane 1, proteins from the 60% to 90% ammonium sulfate fraction; lane 2, DEAE-Sepharose unbound proteins in 50 mM NaCl ionic strength; lane 3, pooled fractions eluted between 0.14 M and 0.25 M NaCl; lanes 4 and 5, ATP-agarose flow-through (lane 4) and eluate (lane 5); lane 6, pooled fractions eluted from the ATP-agarose step of a preparation of b-70 from normal kernels. Arrowheads indicate the positions of the 73-kD and 75-kD proteins.

RESULTS

Isolation of a b-70 cDNA Clone

Antisera to partially purified b-70 were used to screen a λgt11 expression library constructed with kernel RNA. Phage harboring b-70 sequences were identified by further immunoselection, as described in Methods. The largest cDNA clone obtained from this screening (cb70-1) was approximately 1.6 kb. DNA sequence analysis of this b-70 cDNA clone allowed deduction of an open reading frame of 488 amino acids. Comparison of this deduced amino acid sequence with the National Biomedical Research Foundation data bank revealed homology between b-70 and several members of the hsp70 protein family. Figure 1 shows an amino acid comparison of b-70, maize hsp70, mouse BiP, and the yeast BiP homolog KAR2. Homology is found between b-70 and maize hsp70 at 62% of the amino acids encoded by the b-70 cDNA clone. Perhaps more striking are the comparisons of b-70 with BiP and KAR2. These alignments were also found to be identical at 62% of the residues even though the organisms represent extremes of eukaryotic divergence.

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Figure 3. Two-Dimensional Gel Analysis of Affinity-Purified Proteins.

Proteins were subjected to two-dimensional IEF/SDS-PAGE and staining with Coomassie Brilliant Blue. Migration was from basic (right) to acidic (left) for IEF gels and top (cathode) to bottom (anode) for SDS gels. The large arrowheads indicate the 73-kD protein common to all panels; the small arrowheads indicate the 75-kD isoforms specific to f12.

(A) Gel of soluble proteins from f12 kernels. Proteins were extracted with 50 mM Tris-HCl, pH 6.8 at 25°C, 1 mM PMSF.

(B) Gel of soluble proteins from normal kernels.

(C) Gel of pooled b-70 fractions from f12 kernels. Proteins were purified through the ATP-agarose affinity step.

(D) Gel of pooled b-70 fractions from normal kernels.
predicts a stop codon immediately after the HDEL sequences, as would be expected for a BiP homolog (Figure 1, b-70*).

**Purification of b-70**

To purify b-70, we took advantage of its similarity to hsp70 family members and its overproduction in f/2 endosperm. Previous efforts by us and others to purify b-70 failed to remove several persistent contaminating proteins (Galante et al., 1983). However, if b-70 were an hsp70-related protein, it would share the ATP-binding property that has served as an efficient means to purify such proteins (Welch and Feramisco, 1985; Kassenbrock and Kelly, 1989). To test this hypothesis, pooled b-70-enriched fractions eluted from a DEAE-Sepharose column were chromatographed on an ATP-agarose column. The Coomassie-stained proteins are shown in Figure 2. Two proteins of 75 kD and 73 kD, as visualized by SDS-PAGE, coeluted from the affinity column, suggesting that they contain ATP binding sites (Figure 2, lane 5). In an attempt to identify the band...
corresponding to the protein induced in f/2 endosperm, the same purification procedure was conducted with kernels from a normal W64A inbred. After ATP affinity chromatography, a single band of 73 kD was detected in a Coomassie-stained SDS-polyacrylamide gel (Figure 2, lane 6). These results suggested that the 75-kD protein was the one induced in f/2 endosperm. Further support for this identification was provided by SDS polyacrylamide gradient gels (9% to 16%). Only the Coomassie-stained proteins of M, 75,000 showed the positive correlation with the gene dosage of f/2, as expected for b-70 (data not shown; Galante et al., 1983). The purity of the ATP-agarose-eluted proteins (normal and mutant genotypes) was also monitored by two-dimensional gel electrophoresis. Figure 3D shows the purified protein from the normal genotype that migrated as a single polypeptide. In contrast, the protein from the f/2 genotype separated into three distinct species (Figure 3C).

The maize b-70 protein was originally identified by SDS-PAGE and operationally defined as a 70-kD protein induced in the endosperm of f/2 mutants (Galante et al., 1983). However, the results of our two-dimensional gels of water-soluble proteins extracted from normal and f/2 endosperm clearly showed a common 73-kD polypeptide and two apparent isoforms (75 kD) of pl 5.3 and pl 5.4 induced in f/2 endosperm (compare Figures 3A and 3B). The clear resolution of three polypeptides on two-dimensional gels confirmed that the 73-kD species was not affected by the f/2 mutation. Thus, we refer to only the two 75-kD polypeptides as b-70.

To purify further the polypeptides induced in f/2 endosperm, the ATP-agarose-eluted fractions (20 μg to 40 μg) were subjected to reversed phase-HPLC (RP-HPLC). Elution of proteins with a linear acetonitrile gradient resolved two major protein peaks at 35.7 min and 36.8 min, as shown in Figure 4. The identity of the peaks was deduced by comparing the retention times of the f/2 proteins with that of the protein purified from the normal genotype. A significant enlargement of the peak eluting at 35.7 min was observed in the elution profile of the f/2 polypeptides, reflecting the overproduction of b-70 in f/2 mutants. The two 75-kD polypeptides induced in f/2 endosperm were not resolved by RP-HPLC. Whether these two species constitute protein isoforms or differentially processed forms of the same polypeptide is unknown at present.

**Phosphorylation of b-70 in Vivo**

Post-translational modification by phosphorylation and ADP-ribosylation have been demonstrated for mammalian BiP (Carlsson and Lazarides, 1983; Hendershot et al., 1988; Kassenbrock and Kelly, 1989). We attempted to characterize biochemical modifications of b-70 by radiolabeling f/2 endosperm tissue with ^32P. The incorporation of radioactive phosphate into proteins is shown in Figure 5.
Separation by two-dimensional gel electrophoresis showed two strongly phosphorylated proteins of apparent Mr, 73,000 and a single, weakly phosphorylated protein of Mr, 75,000. Immunostaining of the Immobilon-P filter after autoradiography allowed assignment of the phosphorylated 75-kD polypeptide as the pl 5.3 form of b-70. The 73-kD phosphorylated polypeptides were expressed at very low levels in both normal and flfl endosperm and were not recognized by b-70 antisera (Figures 3A and 3B; Figure 5A). The weak labeling of b-70 compared with the amount of Coomassie-stained protein in the gel suggested that either the protein has few phosphorylation sites or a large proportion of the b-70 protein is not phosphorylated.

The pi 5.4 b-70 Species Is not an Unphosphorylated Precursor of the pi 5.3 Polypeptide

We investigated the possibility that the pi 5.3 form of b-70 was a phosphorylated derivative of the pi 5.4 b-70 polypeptide by the following experiments. Endosperm proteins were phosphorylated in vitro by incubation of 30 μg of total protein from normal and mutant endosperm with γ-32P-ATP. An autoradiograph of radiolabeled phosphoproteins separated by two-dimensional gel electrophoresis is shown in Figures 6A and 6B. Incubation of protein extracts with radiolabeled ATP resulted in preferential labeling of the two basic 73-kD proteins (Figure 6, large arrows) as well as the pi 5.3 form of b-70, in agreement with the in vivo results. The absence of a signal corresponding to the pi 5.3 b-70 polypeptide in normal endosperm most likely reflects the decreased expression of b-70 in this genotype. However, the 73-kD basic proteins are labeled to similar extents in both normal and mutant endosperm (compare Figures 6A and 6B). Treatment of the samples with alkaline phosphatase after radiolabeling resulted in the disappearance of the pi 5.3 b-70 radioactive signal but did not alter the pattern of Coomassie blue-stained proteins on a two-dimensional gel (data not shown). Thus, the labeling by γ-32P-ATP must have resulted from covalent incorporation of phosphate moieties onto b-70 rather than noncovalent interaction of the protein with ATP. In addition, because phosphatase treatment does not alter the relative migration and amounts of the two b-70 polypeptides in two-dimensional gels, these polypeptides do not represent phosphorylated and unmodified forms of b-70.

The pi 5.3 b-70 Polypeptide Can Be ADP-Ribosylated in Vitro

We explored the possibility that the observed charge heterogeneity of b-70 was a result of post-translational modification by ADP-ribosylation. To assay the capacity of b-70 to be modified by ADP-ribosylation, we incubated flfl endosperm proteins with 5 μCi of adenylate-32P-NAD⁺ for 15 min at 37°C. Proteins were fractionated by two-dimensional gel electrophoresis. The letters a, b, and c indicate the 73-kD polypeptide, pl 5.3 b-70, and pl 5.4 b-70 polypeptides, respectively.

(A) Autoradiograph of ADP-ribosylated proteins.
(B) Coomassie-stained gel of endosperm proteins.
(C) Coomassie-stained gel of endosperm proteins treated with alkaline phosphatase and snake venom phosphodiesterase I.
endosperm proteins with adenylate-\(^{32}\)P-NAD\(^+\) for 10 min at 37°C and subsequently separated the proteins by twodimensional gel electrophoresis. The in vitro labeling with adenylate-\(^{32}\)P-NAD\(^+\) resulted in incorporation of radioactivity into the pi 5.3 form of b-70, as shown in Figure 7A. Snake venom phosphodiesterase digestion of the labeled proteins resulted in the loss of the radioactive signal (data not shown) and demonstrated conclusively that the incorporation of radioactivity was due to the covalent attachment of ADP-ribose to proteins.

An ADP-ribosylated form of a mammalian BiP has been shown to focus immediately adjacent to the anode side of its precursor protein (Leno and Ledford, 1989). Our labeling results with the pi 5.3 b-70 polypeptide were consistent with the expected focusing position for an ADP-ribosylated form of the pi 5.4 polypeptide. Nevertheless, treatment of the samples with snake venom phosphodiesterase did not result in the expected mobility shift of the pi 5.3 form in two-dimensional gels (data not shown). This result suggests that the pi 5.3 form of b-70 is not the ADP-ribosylated derivative of the pi 5.4 polypeptide.

In light of the dual modifications of the pi 5.3 polypeptide, we also assayed for additive effects of phosphorylation and ADP-ribosylation as a cause for charge heterogeneity of b-70. Enzymatic treatment of the samples with both phosphodiesterase and alkaline phosphatase resulted in a slight shift in the isoelectric focusing (IEF) position of the pi 5.3 polypeptide but did not cause the two b-70 forms to comigrate as a single pi 5.4 species. Instead, both forms were still present (Figure 7C). Thus, the pi 5.4 form is not likely to be the precursor for these post-translational modifications.

Tunicamycin Treatment Induces b-70 in Cell Cultures

Induction of BiP in mammalian cell cultures results from accumulation of abnormal proteins in the ER (Kozutsumi et al., 1988). Such a response is often initiated by treatment with inhibitors of protein glycosylation (Olden et al., 1979). We used tunicamycin, a potent inhibitor of glycosylation in animal and plant cells, to investigate induction of b-70 in cultured maize cells (Hori and Elbein, 1981; Shannon, 1982; Elbein, 1987). Endosperm suspension cells were incubated with 10 \(\mu\)g/mL tunicamycin for 12 hr. Protein extracts and ER fractions from control and tunicamycin-treated cells were separated by two-dimensional gel electrophoresis and blotted onto nitrocellulose. The authenticity of the ER fraction in the sucrose gradient was confirmed by assaying for activity of an ER marker enzyme, NADH-dependent cytochrome c reductase (Larkins and Hurkman, 1978). After transfer to Immobilon-P, b-70 was immunostained with horseradish peroxidase as described in Methods. Because the polyclonal b-70 antisera cross-reacted with several proteins (Figure 5A), we used an immunoaffinity fractionation of b-70 polyclonal antibody to detect b-70 specifically. This clone-selected antibody recognizes only the b-70 polypeptides in two-dimensional immunoblots of total protein from \(f_12\) kernels (data not shown).

Analysis of crude protein extracts as well as the ER protein fraction in the tunicamycin-treated suspension culture cells is shown in Figure 8. This two-dimensional fractionation demonstrated that two proteins were indeed induced. Like the b-70 protein from \(f_12\) endosperm, these proteins migrated with an apparent \(M_r\) of 75,000 (compare Figure 3A and Figure 8B). In addition, both proteins were recognized by the clone-selected antibody (Figures 8C and

![Figure 8. Induction of b-70 Synthesis by Tunicamycin in Cultured Endosperm Cells.](https://academic.oup.com/plcell/article/3/5/483/5984211)

Forty-milliliter cultures of endosperm cells were incubated in the absence (control) and presence of 10 \(\mu\)g/mL tunicamycin for 12 hr. Crude protein extracts and ER fractions were subjected to two-dimensional gel electrophoresis and immunoblotted with b-70 clone-selected antibody. Orientation of gels is the same as in Figure 3. Arrowheads indicate the two isoforms of b-70.
(A) Coomassie-stained gel of proteins from control cells.
(B) Coomassie-stained gel of proteins from tunicamycin-treated cells.
(C) Immunoblot of proteins from control cells.
(D) Immunoblot of proteins from tunicamycin-treated cells.
(E) Immunoblot of ER proteins from control cells.
(F) Immunoblot of ER proteins from tunicamycin-treated cells.
Figure 9. Expression and Subcellular Fractionation of b-70 from BMS Suspension Cultures.

BMS cells were incubated in the absence (control) and presence of 10 μg/mL tunicamycin for 24 hr.

(A) Coomassie-stained gel of protein from cell cultures and endosperm. Letters above lanes denote samples from control (C) and tunicamycin-treated (T) suspension culture cells and normal (N) and mutant (f/2) endosperm. Purified b-70 is used as a marker (b70).

(B) Immunoblot probed with b-70 clone-selected antibody. Lanes contained total protein extracted from control (C) and tunicamycin-treated (T) cells, normal (N) and mutant (f/2) endosperm, and ER proteins from control and tunicamycin-treated cells. PK refers to proteinase K treatment, PK+SDS indicates treatment with proteinase K in the presence of SDS, and (−) denotes absence of protease treatment.

However, a slight difference in pI values was observed and most likely resulted from differences in the A636 background of the cultured cells and the W64A inbred used for kernel studies. In contrast to normal W64A endosperm, uninduced A636 endosperm cultured cells had levels of b-70 that were detectable by Coomassie staining of two-dimensional gels (compare Figures 3B and 3D and 8A). Whether these differences in b-70 levels represent a different signaling pathway or a different metabolic state in kernels and cultured cells is unknown. Immunoblots of the ER fractions further confirmed the induction by tunicamycin and showed that both b-70 polypeptides were associated with membranes in the sucrose gradient (Figures 8E and 8F).

Maize endosperm suspension cultures maintain a very active protein transport process, as judged by the large quantity of acid hydrolases secreted into the medium (Miernyk, 1987). Although unlikely, synthesis of b-70 in the f/2 mutant and in endosperm suspension culture cells may be specific to maize endosperm tissue. To investigate the expression of b-70 in other maize cell types, we examined undifferentiated cultured cells in which we assayed for both the typical response to tunicamycin treatment and recognition by clone-selected antibody as criteria for b-70 identification. The tunicamycin induction in Black Mexican Sweet (BMS) corn cells was performed under the same conditions used for cultured endosperm cells. After cellular fractionation in sucrose gradients, the crude ER fraction was collected and divided into three fractions. One received no treatment, the second was subjected to proteinase K digestion to assay for integrity of the membrane and to remove any contaminating proteins that copurified with the ER, and the third was subjected to proteinase K digestion in the presence of SDS to disrupt membrane structure. After incubation, proteinase K digestion was terminated by addition of phenylmethanesulfonyl fluoride (PMSF), and the samples were immediately processed for SDS-PAGE. Figure 9A shows a Coomassie-stained gel of the protein extracts. Extracts were also immunoblotted and probed with clone-selected b-70 antibody, as shown in Figure 9B. The specificity of the clone-selected antibody enabled us to identify b-70 in the normal W64A genotype in immunoblots overloaded with protein (Figure 9B, lane N).

The protein induced by tunicamycin in BMS suspension cells was similar to b-70 induced in f/2 endosperm. Both proteins had the same electrophoretic mobility in SDS-polyacrylamide gels and they were immunologically related. In addition, the tunicamycin-induced protein was also found in the lumen of ER, as shown by cellular fractionation of suspension cells and treatment with proteinase K (Figure 9B). Separation of proteins from tunicamycin-treated BMS cells in two-dimensional gels resulted in two tunicamycin-induced polypeptides that comigrated with the two forms induced in f/2 endosperm (data not shown).
In our procedure to purify b-70, fl2 endosperm proteins selected by ATP affinity chromatography were resolved into three distinct species. The synthesis of the 73-kD polypeptide, pl 5.2, was not affected by the fl2 mutation, whereas the two 75-kD polypeptides, with pl values of 5.3 and 5.4, were overproduced in fl2 endosperm. Based on this improved resolution of polypeptides in the 70-kD to 75-kD range, we refined the definition of b-70 from that proposed by Galante et al. (1983). We suggest that b-70 should refer to only the two 75-kD polypeptides.

It is unlikely that the 73-kD protein recognized by the polyclonal b-70 antiserum is closely related to the two 75 kD polypeptides. Although all three proteins share ATP-binding properties, clone-selected antisera recognized only the two 75-kD polypeptides (see Figure 8). Nevertheless, this finding does not eliminate the 73-kD protein as a more distantly related member of the hsp70 family.

Although we did not detect b-70 in two-dimensional gels of proteins from normal kernels, it may be expressed at very low levels in this genotype. We did detect low levels of b-70 in uninduced cultured cells (Figure 8) and also in b-70 immunoblots overloaded with total protein from normal endosperm (Figure 9). In addition, b-70 RNA was detectable in both normal and fl2 kernels and in tunicamycin-induced and uninduced BMS suspension cultured cells (Figure 10 and the companion paper, Boston et al., 1991). Recent documentation that a temperature-sensitive mutation in KAR2, the yeast homolog of BiP, could be partially complemented by a mouse BiP cDNA clone suggests that these BiP homologs retained similar biological activities as the two species diverged (Normington et al., 1989). Of residues conserved between BiP and KAR2, 90% are also shared by b-70. Thus, b-70 may also perform similar biological functions. If so, its detection in normal maize kernels and in uninduced cell lines would be expected.

Despite the similarities between b-70 and BiP, some differences are evident. The mammalian BiP is a product of a single gene and usually migrates as a single species on two-dimensional gels. We detected by two-dimensional gel electrophoresis two forms of b-70 in approximately equimolar amounts. The synthesis of both polypeptides was enhanced in fl2 endosperm and in cell cultures treated with tunicamycin. We do not know whether the two apparent isoforms are variants of the same polypeptide or products of different genes. The two forms have very similar pl values (5.3 and 5.4) and similar biochemical properties that suggest they are highly related.

At present, we cannot definitively assign both pl forms of b-70 as BiP homologs. Although other glucose-regulated proteins are localized in ER and induced by glycosylation inhibitors, only BiP has been shown to be phosphorylated (Lee, 1987). This phosphorylation has been proposed as a regulatory control preventing protein function such that only unmodified forms are able to associate with polypeptides (Hendershot et al., 1988). We have ruled

**DISCUSSION**

The hsp70-related proteins constitute a highly conserved family of stress-induced proteins present in both eukaryotic and prokaryotic cells (for review, see Craig, 1985). BiP, a member of an ER-localized hsp70 subfamily first identified in mammals, has been characterized and described as a molecular chaperone involved in mediating the folding and assembly of secretory proteins (Bole et al., 1986; Ellis et al., 1989; Hendershot, 1990).

In this paper, we describe the characterization of a cDNA clone and the corresponding protein b-70. The identification of maize b-70, induced in fl2 endosperm, as a BiP homolog is based on sequence homology, common biochemical properties, and similar responses to stress conditions.

**RNA Gel Blotting of b-70 RNA**

To determine whether the increases in b-70 protein levels reflected changes in b-70 mRNA abundance, we compared b-70 RNA levels using RNA gel blot analysis. Figure 10 shows an RNA gel blot of b-70 RNA from control and tunicamycin-treated BMS maize suspension cultures and normal and fl2 kernels. In contrast to the low levels of b-70 RNA detectable in control cultures, very high levels of b-70 RNA could be detected after tunicamycin treatment. Comparison of b-70 RNA levels in equal amounts of total RNA showed that b-70 RNA levels are greater in the induced cell cultures than in fl2 kernels (Figure 10, lanes 4 and 5).

**Figure 10. RNA Gel Blots of b-70 in Tunicamycin-Treated BMS Cells.**

RNA was isolated from control (C) BMS cells or cells incubated in the presence of tunicamycin (T). Equal amounts of total RNA from normal (N) or fl2 (fl2) endosperm at 20 DAP were included on the same filter to allow direct comparison of the relative induction of b-70 RNA. Lanes 3 and 4 are fivefold overexposures of the portion of the filter containing lanes 1 and 2, respectively. Lanes 3, 4, 5, and 6 are from the same autoradiograph.
out phosphorylation and ADP-ribosylation as the basis for differences in the pI 5.3 and 5.4 forms of b-70 (Figure 7C). Unlike mammalian BiP, b-70 has a potential N-linked glycosylation site, N-Q-S (Figure 1, amino acids 418 to 420). However, neither b-70 polypeptide is glycosylated, as judged by inability to bind to a concanavalin-A-agarose column and failure to be stained by Schiff’s reagent (data not shown).

The molecular basis for the elevated expression of b-70 in f12 endosperm is unknown. Although f12 mutants have a chalky, opaque endosperm that contrasts with the translucent endosperm of normal kernels, the f12 plants are otherwise phenotypically normal. The induction of b-70 RNA levels in tunicamycin-treated cells is consistent with induction of mammalian BiP RNA under similar conditions. Resendez et al. (1988) have identified a 54-nucleotide cis-acting regulatory element important for the induction of BiP in rat and human cell lines. Comparison of b-70 and BiP regulatory mechanisms may provide insight into the molecular effect of the f12 mutation.

Our results suggest that b-70 is a component of the ER lumen because of its resistance to protease treatment. It has been demonstrated by cellular fractionation that b-70 in endosperm is associated with both ER and protein bodies (Galante et al., 1983; Salamini et al., 1985). In f12 endosperm, b-70 was found associated with abnormal protein bodies, and traces of the protein were also reported in protein bodies of normal endosperm (Christianson et al., 1974; Galante et al., 1983). Because the previous report relied solely on one-dimensional SDS-PAGE to separate proteins, the identity of the 70-kD protein in protein bodies of the normal genotype cannot be assigned with certainty. Our results demonstrate clearly the presence of an additional protein that is neither affected by f12 nor localized in ER (Figure 3, Figure 8, and data not shown).

The data presented here are consistent with the assignment of the pI 5.3 form of b-70 as a plant BiP. The increase in b-70 gene expression after tunicamycin treatment and in cells with altered zein aggregation patterns is analogous to BiP induction in mammalian cells and consistent with b-70 having a molecular chaperone role. Together, these results lead us to suggest that b-70 may be a key mediator of protein folding and assembly in maize endosperm.

### Purification of b-70

All purification steps were performed at 4°C. Coarse corn meal from 100 g of 24 DAP frozen kernels was produced with a type 228 Varco inc. coffee mill and stirred for 2 hr with 400-mL of buffer A (50 mM Tris-Cl, pH 6.8 at 25°C, 1 mM PMSF). Cell debris was removed by centrifugation at 16,000g for 10 min. Proteins recovered from a 60% to 90% saturated ammonium sulfate precipitation of the supernatant were resuspended in a minimal volume of buffer B (buffer A + 50 mM NaCl). After extensive dialysis against buffer B, the crude protein extract was fractionated on a DEA-Sepharose CL-6B column (16 x 1.5 cm; Pharmacia, Piscataway, NJ) equilibrated in buffer B and developed with a 240-mL linear gradient of 0.05 M to 0.30 M NaCl in buffer A. Fractions containing b-70 were identified by SDS-PAGE, and those eluting between 0.14 M and 0.25 M NaCl were pooled and dialyzed overnight against buffer C [20 mM Tris-acetate, pH 7.5, 20 mM NaCl, 3 mM MgCl2, 0.1 mM EDTA, 15 mM 2-mercaptoethanol (Welch and Feramisco, 1985)]. The dialysate was applied to an ATP-agarose column (5 x 1 cm; Sigma) equilibrated with the same buffer. The column was washed with 5 bed volumes of buffer C containing 0.5 M NaCl and 5 bed volumes of buffer C alone before elution of the bound proteins with buffer C containing 3 mM ATP. Fractions enriched for b-70 were pooled, concentrated by filter centrifugation (Amicon 30-kD MW exclusion; Amicon, Danvers, MA), and dialyzed against buffer D [50 mM Tris-Cl, pH 7.5 at 25°C, 20 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol].

### RP-HPLC

Dialyzed b-70 fractions from the ATP-agarose column (20 μg and 40 μg of protein from f12 and normal kernels, respectively) were resolved on a Waters HPLC system (Waters Associates, Milford, MA) using a Vydac C4 silica column (4.6 mm x 25 cm, 5-μm particle size; Separations Group, Hesperia, CA). The column was eluted with a 5% to 100% (v/v) linear acetonitrile gradient containing 0.01% (v/v) TFA.

### Preparation of Antiserum

Kernel extracts were fractionated through the DEAE chromatography step described above. Fractions containing b-70, as judged by migration on SDS-polyacrylamide gels, were pooled, mixed in a 1:1 ratio with Freund’s complete adjuvant, and injected (400 μg) subcutaneously into a New Zealand White rabbit. The rabbit was injected again after 2 weeks and subsequently was bled from the ear at 1-week intervals.

### Purification of Clone-Selected Antibodies

To affinity purify monospecific antibody, bacteriophage λgt11 harboring putative b-70 cDNA clones were grown on Escherichia coli strain Y1090 and immobilized on nitrocellulose filters. Filters were subsequently incubated with polyclonal b-70 antiserum to select antibodies specific for β-galactosidase-b-70 fusion proteins. The b-70-specific antibodies were eluted with 0.1 M glycine, pH 2.9, and immediately neutralized (Choi et al., 1987). The eluted

### METHODS

#### Plant Material

Maize (Zea mays) inbred W64A and its near isogenic mutant f12 were grown and self-pollinated at the North Carolina State University Research Unit I, Raleigh, NC, from 1988 to 1990. Ears were harvested 24 days after pollination (DAP) and rapidly frozen in liquid nitrogen. The frozen kernels were shelled onto dry ice and stored at −80°C.
antibody, referred to as clone-selected antibody, was stored in 10 mM Tris-HCl, pH 7.4 at 25°C, 0.14 M NaCl, 0.2% (w/v) NaN₃.

Isolation and Sequencing of b-70 cDNA Clones

An amplified λgt11 cDNA library was prepared and screened essentially as described previously (Huynh et al., 1985). RNA used as starting material was obtained from normal-looking (yellow) 14 DAP kernels from plants (F2/F2) segregating for vp5 in a predominantly TX441 background. The library had a titer of 1.3 x 10⁸ plaque-forming units and was approximately 8.7% nonrecombinant before amplification. Eight potential positives were selected and plaque purified on the basis of these plaque lifts. Positive identification of b-70 cDNA clones was made by incubating clone-selected antibodies with replicate immunoblots of endosperm proteins from normal and F2 maize and purified b-70. Clones that reacted with antibodies to 70-kD proteins that were more abundant in F2 endosperm were assumed to encode b-70.

Sanger dideoxy sequencing of the largest b-70 cDNA clone, cb70-1, was performed on overlapping M13mp18 and M13mp19 subclones using a Sequenase kit (United States Biochemicals, Cleveland, OH; Sanger et al., 1977; Yanish-Perron et al., 1985). Reactions were labeled with adenosine 5'-32P-thiotriphosphate (Amersham, Arlington Heights, IL; specific activity 1,000 Ci/mmol) according to the manufacturer's recommendations. This clone (cb70-1) was performed on overlapping M13mpl8 and M13mpl9 subclones using a Sequenase kit (United States Biochemicals, Cleveland, OH; Sanger et al., 1977; Yanish-Perron et al., 1985). Reactions were labeled with adenosine 5'-32P-thiotriphosphate (Amersham, Arlington Heights, IL; specific activity 1,000 Ci/mmol) according to the manufacturer's recommendations. This clone has been assigned accession no. M59449 in the GenBank database (Bilofsky and Burks, 1988).

PCR Amplification and Cloning of the 3' Region of b-70 mRNA

Membrane-bound polysomal mRNA (0.5 μg) was isolated from 14 DAP W64A/F2 kernels as described previously (Larkins and Hurkman, 1978) and used as a template for reverse transcription with Superscript RNase H-reverse transcriptase ( Gibco) using the poly T-adapter primer (GCAGGATCCTCTAGAGTCGAC) according to the manufacturer’s protocol. PCR was carried out essentially according to Frohman (1990) using 1/100 of the cDNA pool, an internal primer from the b-70 gene (CTTGTCTTTCAGCTTGCGTG), and an adapter primer (GCAGATCCTCTAGAGTCGAC). The amplified product was cleaved with ApaI, which cuts once within the amplified region, and BamHI, which cuts within the adapter primer, and cloned into Bluescript KS/+ by standard techniques. The DNA sequence corresponding to the 3' end of the b-70 mRNA was determined from double-stranded plasmid DNA using the Sequenase system and the 3' adapter primer.

RNA Preparation and Gel Blot Analysis

RNA was isolated from kernels in a W64A background or BMS suspension cultured cells essentially as described by Wadsworth et al. (1988). For RNA gel blot analysis, equal amounts of total RNA were glyoxalated for 60 min at 50°C and fractionated on a 1% agarose, 20 mM phosphate gel (pH 6.9) with recirculation of buffer (McMaster and Carmichael, 1977). The RNA was transferred to nylon membranes (GeneScreen, DuPont-New England Nuclear) by capillary blotting with 20 mM sodium phosphate, pH 6.9, for 14 hr to 16 hr at room temperature. Hybridization probes were radiolabeled with 32P-dCTP by random primed labeling according to the manufacturer’s instructions (Boehringer-Mannheim). Specific activities ranged from 0.8 to 1.3 x 10⁹ cpm/μg. Hybridizations were performed as described for nylon membranes by Galau et al. (1986) for high (Tₛ, -25°C) stringencies. Autoradiography was performed at -80°C using Lightning-Plus intensifying screens (Dupont-New England Nuclear).

In Vitro Post-Translational Modification of b-70

For the two-dimensional electrophoretic fractionation, the in vitro phosphorylation assays were performed with crude protein extracted from normal and mutant endosperm. Equal fresh weights of normal and mutant endosperm were homogenized in 0.2 M KCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 1 mM PMSF, and 0.1 mM DTT for 15 min. Cell debris was removed by centrifugation at 16,000g for 10 min. The supernatant (30 μg) was incubated with 20 mM sodium phosphate, 100 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, and 0.10 μM 32P-ATP (4500 Ci/mmol; ICN, Costa Mesa, CA) for 15 min at room temperature in a final volume of 50 μL. Reactions were stopped with IEF sample buffer and proteins fractionated by two-dimensional gel electrophoresis (O’Farrell, 1975). The gel was stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography at -80°C, using a Lightning-Plus intensifying screen.

Radiolabeling of Endosperm with 32P

Endosperm was dissected from fresh 24 DAP kernels of W64A/F2 and labeled by incubation of the tissue in 0.5 mL of phosphate-free MS (Murashige and Skoog, 1962) medium containing 0.5 mCi 32P (H3PO4 in H2O, HCl free; ICN) for 24 hr. Protein extracts were prepared by homogenization of the endosperm with 0.1 M Tris-HCl, pH 8.0 at 25°C, 0.2 M KCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 1 mM PMSF, and 0.1 mM DTT in the ratio of 1 mg of tissue/2 μL of buffer. To deplete ATP from the homogenate, 1 mM glucose and 10 units of hexokinase (Sigma) were added to the extraction buffer (Hurtley et al., 1989). Proteins were fractionated by two-dimensional gel electrophoresis and electrophoretically transferred to an Immobilon-P, polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The phosphoproteins were visualized by autoradiography at -80°C using an intensifying screen. After autoradiography, b-70 was identified by immunoblotting as described below.

In Vitro ADP-Ribosylation

Proteins were extracted from F2 endosperm with 0.2 M KCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, and 0.1 mM DTT. After centrifugation at 16,000g for 10 min, the supernatant was incubated with 5 μCi of adenosyl-32P-NAD+ for 15 min at 37°C. ADP-ribosylation reactions were stopped by equilibrating the samples with IEF buffer in the ratio of 1:1 (v/v) (O’Farrell, 1975). Proteins were separated by two-dimensional gel electrophoresis and labeled proteins were visualized by autoradiography. After the incubation period, aliquots were also treated with 10 units/μL calf intestinal alkaline phosphatase (Promega Biotec, Madison, WI).
and 0.6 units/μL snake venom phosphodiesterase I from Crotaulus adamanteus (Worthington, Freehold, NJ) for 1 hr at 37°C and analyzed by two-dimensional gel electrophoresis, followed by Coomassie staining.

**Cell Culture and Induction of b-70**

Endosperm cells from the inbred A636 were cultured as described by Shannon (1982). BMS corn suspension cells (obtained from C. Harms, CIBA-GEIGY Corp.) were grown as described previously (Green, 1977). Tunicamycin (Calbiochem, San Diego, CA) was added to cultures at 6 days after passage by dilution of a 5 mg/mL stock in DMSO into normal growth to 0.5 μg/mL. Triplicate cultures were for 12 hr or 24 hr as noted in the figure legends. E.B.P.F., R.L.W. and S.P.M. are recipients of graduate fellowships from the Brazilian Government (CNPq), the McKnight Technology Center and the North Carolina Agricultural Research Foundation, the North Carolina Biotechnology Center, the United States Department of Agriculture, Biomedical Research Support Grant No. RR7071 from the National Institutes of Health, a faculty research initiatives grant from the North Carolina Biotechnology Center, and a support Grant No. RR7071 from the National Institutes of Health. E.B.P.F., R.L.W. and S.P.M. are recipients of graduate fellowships from the Brazilian Government (CNPq), the McKnight Foundation, and the North Carolina Biotechnology Center, respectively.

**Isolation of Crude ER**

Isolation of ER was performed basically as described by Larkins and Hurkman (1978). Briefly, BMS or A636 endosperm suspension cells (4 g) were collected by filtration and homogenized in a Tris extraction buffer [7.2% (w/v) sucrose, 10 mM Tris-HCl, pH 8.5 at 25°C, 10 mM KCl, 5 mM MgCl₂] at 4°C in the ratio of 1 mg of cell/2 μL of grinding buffer. After centrifugation of the homogenate at 500g for 5 min to remove cell debris, the ER-rich organelle fraction was isolated on a discontinuous sucrose gradient consisting of 0.5 M, 1.0 M, 1.5 M, and 2.0 M sucrose layers in extraction buffer. After centrifugation at 80,000g for 30 min at 4°C, the ER fraction was recovered between the 1.0 M and 1.5 M sucrose layers. To eliminate possible protein contamination and to assay for the integrity of the organelles, the ER fraction was treated with 0.15 mg/mL proteinase K (Boehringer Mannheim) for 30 min at 4°C. Protease activity was then inhibited by addition of PMSF to 0.25 mg/mL.

**SDS-PAGE, Two-Dimensional Gel Electrophoresis, and Immunostaining**

SDS-PAGE was performed as described by Laemmli (1970) and two-dimensional gel electrophoresis as described by O'Farrell (1975). The pH gradient of the isoelectric focusing gels was determined by equilibrating 0.5-cm gel slices in 25 mM KCl and measuring the pH of the solutions with an MI415 electrode (Microelectrodes Inc., Londonderry, NH). For immunostaining, proteins were electroblotted from 10% SDS-polyacrylamide gels to nitrocellulose using a semidry blotting apparatus (PolyBlot, American Bionetics, Hayward, CA) according to the manufacturer’s instructions. Immunoblot analysis, in which b-70 polyclonal antibody was used at 1:2000 dilution, was performed using a horse radish peroxidase color reaction as described previously (Lending et al., 1988).

Protein concentration was determined as described by Hill and Straka (1988) for samples lacking DTT. For those samples containing DTT, protein concentrations were determined according to Bradford (1976).

**REFERENCES**


**ACKNOWLEDGMENTS**

We thank Ross Whetten and David O’Malley for use of their RP-HPLC system, Jeff Rollins for help with the two-dimensional gel electrophoresis, Christian Harms for the BMS suspension culture, and Richard Burgess, Linda Hendershot, Sam Levings, Steve Spiker, and Evelyn Kawata for critical reading of the manuscript. We are grateful to Hank Bass for helpful comments and discussions. This work was supported by Grant 50-37261-5618 from the United States Department of Agriculture, Biomedical Research Support Grant No. RR7071 from the National Institutes of Health, a faculty research initiatives grant from the North Carolina Biotechnology Center and the North Carolina Agricultural Research Service. E.B.P.F., R.L.W. and S.P.M. are recipients of graduate fellowships from the Brazilian Government (CNPq), the McKnight Foundation, and the North Carolina Biotechnology Center, respectively.

Received February 19, 1991; accepted February 25, 1991.


