The Plant Cell, Vol. 4, 821–829, July 1992 © 1992 American Society of Plant Physiologists

# The 70-Kilodalton Heat Shock Cognate Can Act as a Molecular Chaperone during the Membrane Translocation of a Plant Secretory Protein Precursor

**Jan A. Miernyk,[a,1] Nicholas B. Duck,[b] Robert G. Shatters, Jr.,[a,2] and William R. Folk[b]**

[a] Seed Biosynthesis Research Unit, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research, 1815 North University Street, Peoria, Illinois 61604

[b] Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, Missouri 65201

**When a model secretory precursor was synthesized in vitro and analyzed by rate-zonal sedimentation, it appeared to be associated with other proteins present in a wheat germ extract. At least one of the associated proteins is a member of the 70-kD family of stress proteins. It was possible to immunoprecipitate the secretory precursor with anti-heat shock cognate 70 (Hsc70) antibodies in the absence but not in the presence of ATP, suggesting that the association was specific. ATP-sensitive association is one diagnostic characteristic of molecular chaperone-type proteins. Increasing incubation temperature decreased the amount of precursor associated with Hsc70. A method was developed for the removal of Hsc70 from a wheat germ in vitro translation mixture by immunoprecipitation. Cotranslational translocation and processing of the secretory precursor by maize endosperm microsomes were inefficient in the Hsc70-depleted system but were greatly stimulated by addition of purified preparations of various heat shock 70 proteins (Hsp70s). Cytosolic Hsc70 from maize endosperm was capable of autophosphorylation in vitro. Phosphorylated Hsc70 was much less efficient in promoting membrane translocation of the secretory precursor. These results suggest that chaperone function in vivo could be regulated by phosphorylation.**

## INTRODUCTION

Molecular chaperones are proteins that mediate the translocation, folding, and assembly of other proteins but are not themselves components of the final structures (Ellis et al., 1989). Molecular chaperones act to forestall misfolding of proteins, preventing the formation of biologically incorrect structures. In the cases of precursor proteins, molecular chaperones help to maintain the polypeptide chain in an unfolded, translocation competent form until transport through the membrane has been completed (Rothman, 1989). The mechanisms by which molecular chaperones control protein folding are not well understood. It is thought that the chaperones recognize and bind to polypeptide surface features that will interact in the final conformation, shielding them until the appropriate time for exposure (Ellis and Hemmingsen, 1989). It has also been shown that molecular chaperones have an intrinsic ATPase activity (Pelham, 1989) and that at least a portion of chaperone function could be in acting as ATP-dependent protein "unfoldases."

Multiple classes of molecular chaperones have been described including several stress-related proteins and the bacterial trigger factor and Sec proteins. The chaperonins, or members of the 60-kD family of stress proteins, have been the most studied molecular chaperones. In higher plants, the role of the plastid chaperonins in assembly of Rubisco has been studied extensively (Gatenby and Ellis, 1990). It has also been demonstrated that chaperonins can interact with a wide variety of precursor proteins imported by chloroplasts (Lubben et al., 1989) and that the chaperonins are present in several cellular compartments (e.g., Grimm et al., 1991). These observations suggest that there are multiple roles for the multiple molecular chaperones in plant cells.

Although the SecB and GroEL proteins appear to be the primary molecular chaperones of bacterial secretory proteins (reviewed by Kumamoto, 1991), there have also been reports of the participation of Hsp70 as a chaperone (e.g., Phillips and Silhavy, 1990). It is well established that Hsp70 acts as a molecular chaperone in the translocation of precursor proteins across the endoplasmic reticulum (ER) membrane of the yeast Saccharomyces (Chirico et al., 1988; Deshaies et al., 1988). Furthermore, Zimmermann et al. (1988) reported that the addition of Hsp70 stimulates the in vitro translocation and processing of a viral precursor protein by canine pancreatic microsomes. We have recently developed an effective maize endosperm-derived system for in vitro analysis of the translocation and processing of secretory protein precursors and have

[1] To whom correspondence should be addressed.

[2] Current address: Agronomy Department, Newell Hall, University of Florida, Institute of Food and Agricultural Sciences, Gainesville, FL 32611.

begun to dissect the components of this system at the molecular level. The experiments described herein examine the potential role(s) of cytoplasmic Hsc70 as a molecular chaperone for plant secretory protein precursors.

## RESULTS

### Sedimentation Analysis of a Secretory Precursor Synthesized in Vitro

Figure 1 shows that when translation reactions were depleted of ATP and then analyzed by rate-zonal sedimentation on linear sucrose gradients, the truncated 26312-D form of a maize plasma membrane-located, receptor-protein kinase precursor (pre-MRKΔXhoI) migrated much further into the gradients than would be predicted based upon migration of the standard proteins carbonic anhydrase ($M_r$ 29000) and bovine serum albumin ($M_r$ 68000). In addition, it was observed that at least some of the wheat germ Hsc70 also migrated further than predicted. Whereas the peaks of pre-MRKΔXhoI and Hsc70 in the gradients are not coincident, they do overlap to a significant extent (Figure 1).

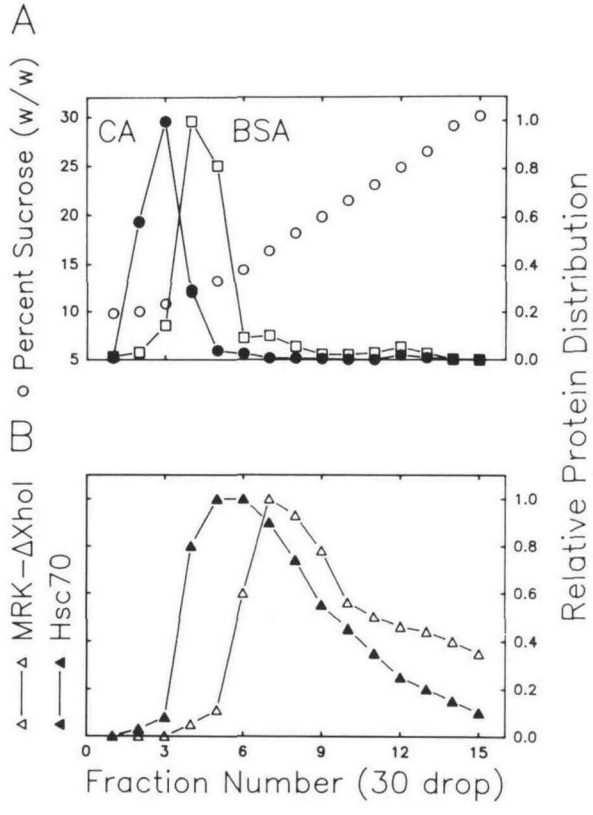


**Figure 1.** Analysis of in Vitro Translation Mixtures by Rate-Zonal Sedimentation.

**(A)** Proteins were separated by centrifugation for 20 hr at 36500 rpm on 12-mL linear 10 to 30% (w/w) sucrose gradients. Samples were TCA precipitated and then analyzed by SDS-PAGE. The standard proteins carbonic anhydrase (CA) and bovine serum albumin (BSA) were detected by Coomassie blue staining plus densitometry. Sucrose concentrations were determined by refractometry.

**(B)** The MRKΔXhoI precursor was quantitated by radioanalytic imaging of in vitro translation products. Hsc70 was detected by protein gel blotting of samples followed by densitometry.

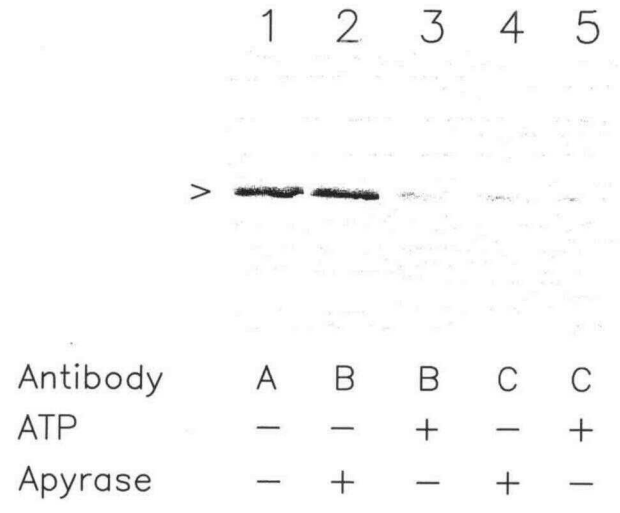


**Figure 2.** Immunoprecipitation of Pre-MRKΔXhoI.

RNA was translated in vitro using a wheat germ–derived system. The in vitro translation mixtures were incubated for 20 min at room temperature with either apyrase or 3 mM MgATP, followed by addition of the antibodies. The immunoprecipitates were washed, dissociated, then analyzed by SDS-PAGE. The antibodies used were: A, anti-MRK N-terminal peptide; B, anti-Hsc70; C, anti-MRK C-terminal peptide. The epitope defined by the synthetic C-terminal peptide is not present in the truncated MRKΔXhoI protein. The position of authentic pre-MRKΔXhoI is indicated by the carat to the left of lane 1.

### Complex Formation in Vitro

Figure 2, lane 1 shows that pre-MRKΔXhoI could be immunoprecipitated from in vitro translation mixtures using antibodies raised against a synthetic peptide corresponding to a sequence located within the mature N-terminal domain of the protein. The precursor could also be immunoprecipitated with anti-Hsc70 antiserum in the absence but not in the presence of ATP (Figure 2, lanes 2 and 3). No in vitro product was immunoprecipitated when an irrelevant antibody, one raised against a synthetic peptide located within the C-terminal domain of the full-length maize receptor-protein kinase (MRK) protein, was used (Figure 2, lanes 4 and 5). Whereas the precursor could not be immunoprecipitated with anti-Hsc70 antibodies in the presence of 3 mM ATP, it could be precipitated equally

well in the absence of ATP or in the presence of a nonhydrolyzable ATP analog 5′-adenylylimidodiphosphate at 3 mM (data not shown).

The avidity of the association between Hsc70 and pre-MRK was evaluated by immunoprecipitation at various temperatures. Figure 3 shows that at 4°C more than 90% of pre-MRK was associated with Hsc70, as judged by immunoprecipitation with anti-Hsc70 antiserum. The proportion of pre-MRK that could be immunoprecipitated with anti-Hsc70 antiserum decreased with increasing temperature. Virtually no pre-MRK was precipitated at 37°C (Figure 3). In contrast, immunoprecipitation of the precursor using anti-MRK N-terminal peptide antibodies was relatively insensitive to increasing temperature (Figure 3).

### Removal of Hsc70 from in Vitro Translation Mixtures

The highly conserved immunoreactivity of plant Hsc70s can be seen in Figure 4. Both maize and wheat proteins cross-reacted with anti-tomato Hsc70 antibodies (Figure 4, lanes 1 and 2). Hsc70 is relatively abundant in the commercial wheat germ extract translation system. It was, however, possible to deplete the extracts of Hsc70 using specific antibodies (Figure 4, lane 3). Depletion of Hsc70 did not result in any significant reduction in the efficiency of translation of commercial brome mosaic virus RNA or in vitro transcribed pre-MRKΔXhoI RNA (data not shown). A positive signal was observed when purified maize endosperm microsomes were probed with anti-Hsc70 antibodies, and limited proteolysis of intact vesicles failed to reduce this signal (Figure 4, lanes 4 and 5). Hsc70 could be released from the microsomes by a mild alkaline treatment known to release lumenal proteins (Figure 4, lanes 6 and 7).

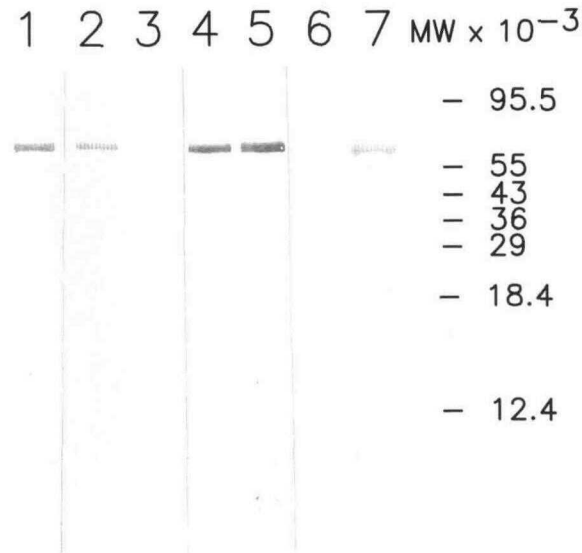


**Figure 4.** Protein Gel Blot Analysis for the Detection of Hsc70s.

Sample proteins were separated by SDS-PAGE using 10% (T) acrylamide gels, then transferred to nitrocellulose membranes. The membranes were incubated with anti-tomato Hsc70 antiserum diluted 1:500, followed by washing and incubation with alkaline phosphatase–conjugated goat anti–rabbit IgG. Samples analyzed are as follows: lane 1, purified Hsc70 from maize endosperm cultures; lane 2, a wheat germ extract translation mixture; lane 3, a wheat germ extract depleted of Hsc70; lane 4, microsomal membranes isolated from maize endosperm cultures; lane 5, maize microsomes after limited proteolysis; lane 6, maize microsomes after mild alkaline treatment; lane 7, lumenal proteins released by alkaline treatment. The positions of standard proteins are indicated to the right of lane 7.

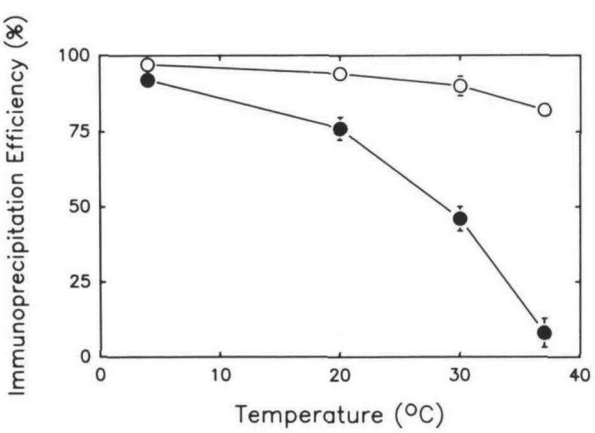


**Figure 3.** Effect of Increasing Temperature on Immunoprecipitation of MRKΔXhoI Precursor.

Antibodies used were ○, anti-MRK N-terminal peptide; ●, anti-Hsc70. Both immunoprecipitation and washing of the precipitates were conducted at the indicated temperatures. Total pre-MRKΔXhoI was determined by TCA-precipitating half of each translation reaction followed by SDS-PAGE and radioanalytic imaging. Error bars indicate SE ($n$ = 4).

### Hsc70 Stimulation of in Vitro Precursor Translocation and Processing

Cotranslational translocation and processing of pre-MRKΔXhoI were relatively inefficient when conducted using a wheat germ extract depleted of Hsc70, as shown in Figure 5A, lanes 1 and 2. However, by titrating the reactions with purified maize endosperm Hsc70, it was possible to restore translocation and processing of the precursor (Figure 5A, lane 3). The stimulation of translocation and processing was saturable (Figure 5B). Table 1 presents the $K_m$ value for maize endosperm Hsc70: approximately 4 μg per reaction. This corresponds to a concentration of approximately 80 μM. The addition of equimolar concentrations of bovine brain Hsc70, the *Escherichia coli* DnaK protein, or the pea chloroplast Cpn60 protein resulted in 81, 47, and 16% as much processing as was estimated for saturating concentrations of maize endosperm Hsp70. Approximately

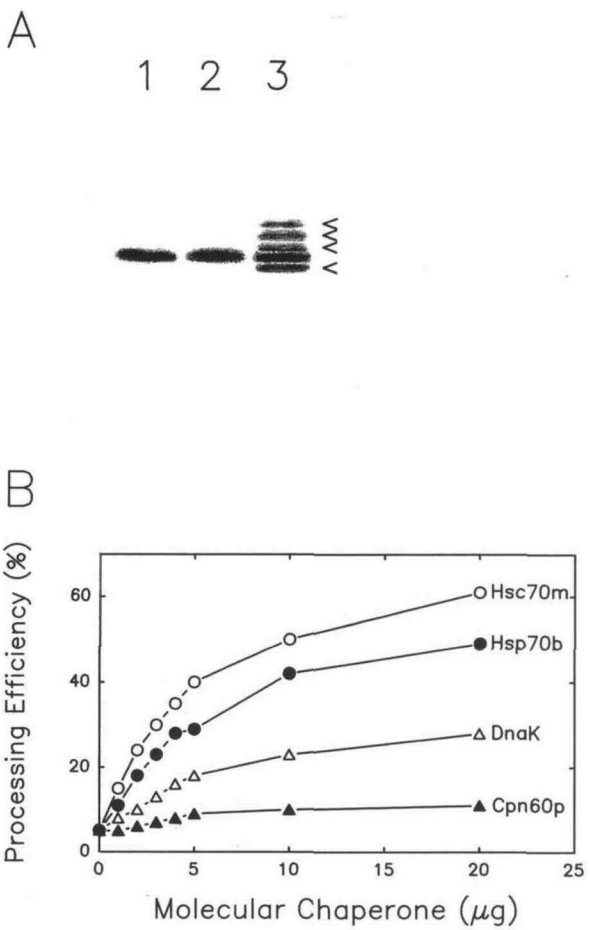


**Figure 5.** Translocation and Processing of the Secretory Precursor in the Absence and Presence of Hsc70.

**(A)** Analysis by SDS-PAGE plus radioanalytic imaging of in vitro synthesized pre-MRKΔXhoI. Lane 1, pre-MRKΔXhoI synthesized in a control wheat germ extract; lane 2, pre-MRKΔXhoI synthesized in an Hsc70-depleted wheat germ extract plus maize endosperm microsomal membranes; lane 3, pre-MRKΔXhoI synthesized in an Hsc70-depleted wheat germ extract plus maize microsomal membranes plus 15 μg of purified maize Hsc70. The positions of the protease-resistant processing intermediates are indicated by carats at the right.

**(B)** The effect of increasing amounts of added molecular chaperones on precursor translocation and processing. The chaperones used are as follows: ○, maize endosperm Hsc70; ●, bovine brain Hsp70; △, *E. coli* DnaK; ▲, pea chloroplast Cpn60.

5% of the precursor was processed in the absence of additional chaperones.

### Autophosphorylation of Cytosolic Hsc70

Hsc70 from maize endosperm cultures, purified by anion exchange, affinity, and gel permeation chromatography, was phosphorylated when incubated in vitro with $\gamma$-$^{32}$P-ATP at pH 6.0 with 20 mM $CaCl_2$, as shown in Figure 6A. Mild acid hydrolysis plus two-dimensional thin-layer electrophoresis revealed phosphothreonine as the only labeled residue (Figure 6B).

### Phosphorylated Hsc70 Is Not an Efficient Molecular Chaperone in Vitro

Purified maize endosperm Hsc70 was phosphorylated in vitro using unlabeled MgATP and then separated from other reaction products by passage through a small column of Sephadex G-25. The peak Hsc70-containing fractions were combined and freeze dried. Translocation and processing of the secretory precursor using an Hsc70-depleted system were conducted as described previously. The reactions were then supplemented with either phosphorylated or nonphosphorylated Hsc70. In the experiment presented in Figure 7, there was no detectable translocation or processing of the precursor in the absence of Hsc70. In the presence of a saturating concentration of Hsc70, 59% of the precursor was processed, whereas only 16% was processed when reactions were supplemented with phosphorylated Hsc70.

## DISCUSSION

Pre-MRKΔXhoI synthesized in a wheat germ extract had a much greater sedimentation coefficient than expected for a monomeric, nonassociated polypeptide (Figure 1). This observation suggested the possibility of a complex between the secretory precursor and components of the wheat germ extract. Some of the wheat germ Hsc70 also migrated further into sucrose gradients than expected. Although peaks corresponding to the precursor and Hsc70 are not coincident, they do overlap. Lecker et al. (1989) reported sharp, coincident peaks during sedimentation analysis of complexes between secretory precursors and bacterial chaperones. In these studies, however, a single species of pure precursor was diluted into

**Table 1.** Kinetic Analysis of the Stimulation of Translocation and Processing of the MRKΔXhoI Precursor by Increasing Concentrations of Various Molecular Chaperones

| | $K_m$ | $V_{max}$ |
|---|---|---|
| Chaperone | μg Assay$^{-1}$ | % Processed |
| Maize Hsc70 | 4.2 ± 0.5 | 73 |
| Bovine Hsp70 | 4.7 ± 0.8 | 59 |
| *E. coli* DnaK | 4.4 ± 1.2 | 34 |
| Pea Cpn60 | 1.7 ± 1.1 | 12 |

Analyses were conducted by iterative curve fitting using nonlinear regression (Garland and Dennis, 1980).

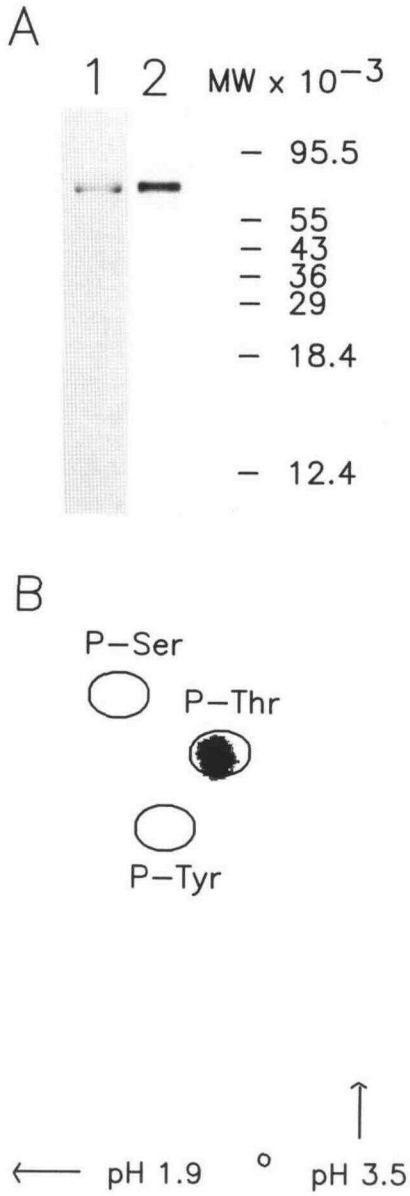


**Figure 6.** *Autophosphorylation of Maize Endosperm Hsc70 in Vitro.*

**(A)** Highly purified Hsc70 from maize endosperm cultures was incubated in vitro at pH 6.0 with $\gamma$-$^{32}$P-ATP plus 5 mM $CaCl_2$. An equal volume of ice-cold 20% (w/v) TCA was added to the incubation mixture. The acid-insoluble material was then analyzed by SDS-PAGE plus radioanalytic imaging. Lane 1, maize endosperm Hsc70 stained with Coomassie blue; lane 2, radioanalytic image of lane 1. The positions of standard proteins are indicated at the right.

**(B)** After phosphorylation in vitro, samples of maize endosperm Hsc70 were subjected to mild acid hydrolysis, and the products were separated by two-dimensional thin layer electrophoresis. The positions of authentic standards were determined by spraying the plates with ninhydrin. The positions of radioactive samples were determined by radioanalytic imaging.

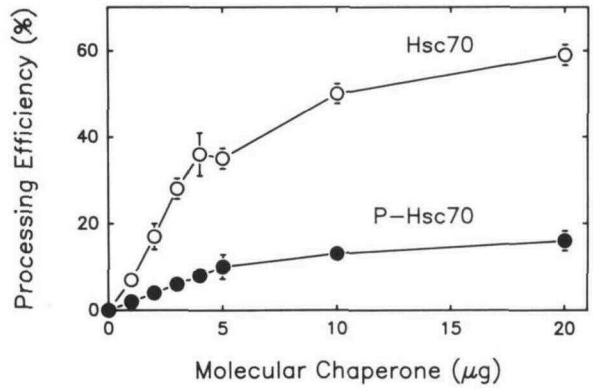


**Figure 7.** Effect of Phosphorylation on the Translocation and Processing of a Secretory Precursor.

The translocation and processing experiments were conducted as described in the legend to Figure 5. Hsp70-depleted reactions were supplemented with maize endosperm Hsc70, either before (○) or after (●) autophosphorylation of the molecular chaperone. Error bars indicate SE ($n$ = 4).

a solution containing a pure chaperone. Interpretation of the behavior of a precursor synthesized in an unfractionated wheat germ extract is much more complicated. There have been reports of complex formation between two or more types of molecular chaperones and precursor proteins (Mizzen et al., 1991; Perdew and Whitelaw, 1991). In addition, complexes between Hsp70, Hsp90, and at least three other proteins have been isolated from mammalian cell cytosol (Perdew and Whitelaw, 1991). Our results are consistent with a complex containing a minimum of two components, Hsc70 and the secretory precursor. When a sample of an in vitro translation mixture was incubated with 3 mM MgATP prior to sedimentation analysis and then loaded onto a linear sucrose gradient also containing 3 mM MgATP, pre-MRKΔXhoI migrated into the gradient to the same position as the carbonic anhydrase protein used as a standard (data not shown).

It was possible to isolate the protein kinase-Hsc70 complex by immunoprecipitation of the in vitro product using anti-Hsc70 antibodies (Figure 2). The complex between precursor and Hsc70 was stable only at low temperature and in the absence of ATP. Both of these factors are characteristic of molecular chaperones. It has been suggested that the ATPase activity indigenous to molecular chaperones is the result of an ATP-dependent protein-unfolding activity (Pelham, 1989). Our results with a nonhydrolyzable ATP analog indicate that ATP hydrolysis is necessary for dissociation of the complex. It appeared that virtually all of the pre-MRKΔXhoI synthesized in vitro was associated with Hsc70, as judged by immunoprecipitation at 4°C (Figure 3). The proportion of pre-MRK that could be immunoprecipitated with anti-Hsc70 antibodies decreased with increasing temperature. It has previously been noted that the association between target proteins and molecular chaperones is temperature sensitive (e.g., Machamer et al., 1990; Earl

et al., 1991). Although this type of association is in many instances transient, it seems likely that a complex can be stabilized at reduced temperatures.

The anti-tomato Hsc70 antibodies proved to be an effective tool for in vitro analysis of the processing of secretory protein precursors, despite being used in an otherwise all-monocot system. This was not unexpected, given the highly conserved structures of all members of the 70-kD family of stress proteins analyzed thus far (Vierling, 1991). Both purified maize endosperm Hsc70 and the wheat germ extracts gave strong positive signals when probed with the tomato antibodies (Figure 4, lanes 1 and 2). It was possible to deplete the wheat germ extracts of Hsc70 by the relatively gentle procedure of immunoaffinity chromatography (Figure 4, lane 3). From densitometry of protein gel blots, it was estimated that less than 30% of Hsc70 remained after a single passage through the antibody column and less than 10% remained after a second passage. Sample dilution was approximately 20% after the immunoremoval, and there was no obvious decrease in the efficiency of incorporation of $^{35}$S-methionine into either standard or experimental proteins.

Maize endosperm microsomes also gave a positive signal when probed with the anti-Hsc70 antibodies (Figure 4, lane 4). Because translocation-competent microsomes were prepared by a combination of rate-zonal sedimentation and gel permeation chromatography, it seemed unlikely that cytoplasmic Hsc70 was adsorbed onto the surface of the membranes. Nevertheless, this possibility was addressed directly by limited proteolysis of the microsomes. A treatment known to remove surface-adsorbed proteins (proteinase K at 0.3 mg mL$^{-1}$ for 45 min at 4°C) failed to significantly reduce the signal (Figure 4, lane 5). The Hsc70 signal could, however, be greatly reduced by a mild alkaline treatment developed to deplete microsomes of soluble lumenal proteins (Paver et al., 1989) (Figure 4, lanes 6 and 7). Thus, it appears that the Hsc70-reactive protein present in maize endosperm microsomes is located not on the surface but within the vesicle lumen. It seems most likely that this microsomal Hsc70-reactive protein is the immunoglobulin binding protein (BiP) homolog known to be present in maize endosperm (Boston et al., 1991). This conclusion is strengthened by the observation that a maize microsomal protein having the same electrophoretic mobility as microsomal Hsc70 gives a positive signal when probed with anti-HDEL antibodies, whereas cytosolic Hsc70 does not (data not shown). The ER lumenal Hsc70 homolog is thought to be essential for the translocation of secretory precursors (Vogel et al., 1990), but the role of the chaperone in this compartment is distinct from that played by cytoplasmic Hsc70.

When pre-MRKΔXhoI was synthesized in an Hsc70-depleted wheat germ extract supplemented with maize endosperm microsomes, there was little conversion of the precursor to the various processing intermediates (Figure 5A, lane 2). When the Hsc70-depleted translation mixtures were supplemented with purified Hsc70, however, there was substantial processing of the precursor to yield a ladder of products, as indicated by the carats to the right of Figure 5A, lane 3. The ladder of radioactive products corresponds to MRK with the signal sequence removed (the band with the greatest electrophoretic mobility) and, in order of decreasing mobility, MRK with the signal sequence removed plus 1, 2, or 3 high-mannose–type glycan side chains added. Processing efficiency was determined by adding the contribution of each of the four products that were translocated into the lumen of the microsomal vesicles (determined by protection from digestion by proteinase K) and then dividing the sum by the total radioactivity present in an equivalent reaction without added microsomes.

Our results are similar to those previously reported for yeast (Chirico et al., 1988; Deshaies et al., 1988) and mammalian (Zimmermann et al., 1988) in vitro experiments. The degree of dependence of complex formation upon Hsc70 addition is clearer in our experiments than in the other reports. However, only in our experiments was there specific prior removal of the chaperones. Deshaies et al. (1988) additionally demonstrated the requirement for Hsc70 in vivo. When the level of yeast Hsc70 was reduced by gene disruption, there was an accumulation of prepro-α-factor in the cytosol.

The stimulation of in vitro translocation and processing by Hsc70 was saturable (Figure 5B), and a $K_m$ of approximately 4 μg per reaction was estimated (Table 1). Both bovine Hsp70 and the *E. coli* Hsp70 homolog, the DnaK protein, promoted precursor translocation and processing, although neither resulted in a processing efficiency as high as observed with maize endosperm Hsc70. In light of our observations, it is possible to reevaluate some of the results obtained by Zimmermann et al. (1988). They studied the import of M13 procoat synthesized in an *E. coli* extract by canine pancreatic microsomes. Import was stimulated by the addition of purified rat Hsp70. It is likely that the in vitro precursor is associated with the DnaK protein, which would promote some translocation into the canine microsomes but would not be as efficient as with mammalian Hsp70. Whereas the various 70-kD proteins promoted processing to significantly different extents in the plant-derived in vitro system, the $K_m$ values for each were the same. It could be that a similar number of chaperone molecules bind to each precursor, but their efficiency in promoting a translocation competent conformation in the precursor varies in relation to their system homology. We observed little processing of the precursor in reactions supplemented with the unrelated pea chloroplast molecular chaperone, the Cpn60 protein (Figure 5B).

Whereas our results were obtained with a single plant precursor protein in an in vitro translocation and processing system, they are completely consistent with the proposal that Hsc70 serves as a molecular chaperone in the translocation of secretory protein precursors. Thus, three cytoplasmic chaperones of secretory precursors have been identified: the ribosome, signal recognition particle, and Hsc70. It is not clear precisely when during the process of protein translocation Hsc70 performs as a molecular chaperone. It is known, however, that Hsc70 can interact with nascent polypeptides (Beckmann et al., 1990). For some proteins, or under some circumstances, polypeptide chain elongation can exceed the rate of membrane translocation. Under these circumstances, Hsc70 might be required to prevent premature folding of the precursor. The

relative participation of each chaperone probably varies depending upon the primary-sequence–dependent physicochemical properties of a given precursor protein. We noted that efficient translocation and processing of the *E. coli* precursor protein pre-β-lactamase by maize endosperm microsomes required less than half as much maize Hsc70 as was necessary with pre-MRKΔXhoI (data not shown).

It was observed initially that the *E. coli* Hsp70 homolog was phosphorylated in vivo (Zylicz et al., 1983) and that eukaryotic mitochondrial Hsp70 homologs could be phosphorylated in vitro in a calcium-stimulated reaction (Leustek et al., 1989). It was demonstrated recently that the bacterial protein autophosphorylates at Thr-199 and phosphorylation greatly reduces the intrinsic ATPase activity (McCarty and Walker, 1991). Highly purified maize endosperm Hsc70 is also capable of autophosphorylation (Figure 6A) on a Thr residue (Figure 6B). It should be noted that our purified preparations of Hsc70 could contain representatives of all members of the Hsp70 family that are constitutively expressed in maize endosperm, including the BiP homolog (Boston et al., 1991). Based on the homology of the sequences flanking Thr-199 of *E. coli* DnaK, it was proposed that autophosphorylation of human Hsp70 and bovine Hsc70 occurs at Thr-204 (McCarty and Walker, 1991). The corresponding sequences suggest phosphorylation at Thr-208 of maize Hsc70 (Rochester et al., 1986), although this remains to be demonstrated.

We propose that phosphorylation of maize endosperm Hsc70 is a potential regulatory mechanism during chaperone function. Phosphorylation of the bacterial Hsp70 homolog greatly reduced, and removal of Thr-199 by site-directed mutagenesis virtually eliminated, the endogenous ATPase activity (McCarty and Walker, 1991). Incubation with the nonhydrolyzable ATP analog 5′-adenylylimidodiphosphate did not promote release of Hsc70 from the complex with the secretory precursor, indicating that hydrolysis of ATP and not simply nucleotide binding is essential. We therefore reasoned that prior autophosphorylation of Hsc70 should abolish molecular chaperone activity. As shown in Figure 7, there was a sevenfold decrease in chaperone function with phosphorylated Hsc70. This is a reasonably good correspondence, considering our lack of knowledge of the extent of phosphorylation in vitro. It is possible that Hsc70 plays multiple roles during membrane translocation of secretory precursors and that not all of these roles require ATP hydrolysis. This hypothesis can be tested through the use of mutant proteins such as those described by McCarty and Walker (1991).

## METHODS

### Molecular Chaperones

The *Escherichia coli* DnaK protein was obtained from Epicenter Technologies, Madison, WI, and bovine brain Hsp70 was obtained from StressGen Biotechnologies Corp. (Sidney, British Columbia, Canada). Hsc70 was purified from 7-day-old maize endosperm cultures (Miernyk, 1987), essentially as described by Welch and Feramisco (1985). One kilogram fresh weight of tissue yielded approximately 4.3 mg of Hsc70. For autophosphorylation, samples of highly purified maize endosperm Hsc70 were incubated in vitro with 2 mM MgATP using the conditions described by Leustek et al. (1989). The Cpn60 protein was purified from a chloroplast-enriched fraction prepared from 10-day-old pea seedlings, as described by Hemmingsen and Ellis (1986). One kilogram fresh weight of tissue yielded approximately 3.5 mg of Cpn60 protein.

### Plant Materials

The maize (*Zea mays* inbred A636) endosperm cultures were described by Miernyk (1987) and Miernyk and Sturch (1991). Microsomes were prepared from the A1 cell line 4 days after transfer to fresh medium. Translocation-competent microsomes were prepared by a modification of the previously described procedure (Riedell and Miernyk, 1988). A complete description of the modifications will be presented elsewhere (R. G. Shatters, Jr. and J. A. Miernyk, manuscript in preparation). Mild alkaline treatment of microsomes was carried out as described by Paver et al. (1989).

### Antibody Preparation

A portion of the 3′ coding region of a previously described tomato Hsc70 cDNA (Lin et al., 1991) was cloned into the bacterial expression vector pGEX-2T (Pharmacia LKB Biotechnology) to produce a glutathione S-transferase-Hsc70 fusion protein. The Hsc70 cDNA was linearized by digestion with PstI, the 3′ overhanging sequences removed using the exonuclease activity of T4 DNA polymerase, and an approximately 900-bp fragment released by digestion with EcoRI. The purified cDNA fragment was cloned into SmaI- and EcoRI-digested pGEX-2T. To move the Hsc70 sequence into the correct reading frame, the construct was linearized by digestion with BamHI and the 5′ overhanging sequences were filled in using the Klenow fragment of DNA polymerase I. The linearized plasmid was then blunt-end ligated and used to transform *E. coli* JM101. Glutathione S-transferase-Hsc70 fusion proteins were purified to homogeneity by passing bacterial cell lysates, prepared in phosphate-buffered saline (PBS) plus 1% (v/v) Triton X-100, over a column of glutathione-agarose. The column was washed with PBS, and the fusion protein was eluted with 50 mM glutathione in 50 mM Tris-HCl, pH 8.0. The affinity-purified fusion protein was used to produce rabbit polyclonal antibodies.

Rabbit polyclonal antibodies against the synthetic peptides, NKTIVWSANPDR, corresponding to residues 83 to 94, and KLVRMLSAKLEG, corresponding to residues 750 to 761 of the primary sequence predicted by the MRK open reading frame, were supplied by J. C. Walker and V. Counihan (Division of Life Sciences, University of Missouri, Columbia).

### In Vitro Transcription, Translation, Translocation, and Processing

Topogenic analysis of the MRK cDNA resulted in the prediction of a large protein with four distinct domains: a signal sequence, an extracellular domain having homology with the S-locus proteins of *Brassica*, a membrane-spanning helical region flanked by a stop-transfer signal, and an intracellular serine/threonine kinase domain (Walker and Zhang, 1990). We have begun in vitro studies of the functional role of each predicted domain. A truncated version of the protein MRKΔXhoI

was created by deletion of the coding region downstream of a unique internal XhoI site. The truncated protein lacks the membrane anchor domain and the downstream protein kinase domain. It thus represents a model soluble secretory precursor.

The plasmid pROB-11, which encodes the MRKΔXhoI precursor protein, was transcribed in vitro using T7 RNA polymerase, exactly as described by Shatters and Miernyk (1991). The MRKΔXhoI precursor protein was synthesized in vitro using a minus methionine wheat germ–derived translation mixture from Promega and L-$^{35}$S-methionine (ICN, Irvine, CA). Cotranslational translocation and processing of the precursor by maize microsomal vesicles were analyzed using the methods of Shatters and Miernyk (1991).

Anti-Hsc70 antibodies were purified from rabbit antiserum using an Amicon MAC protein-G capsule. Purified antibodies were then immobilized using a ProtOn 2 kit from Multiple Peptide Systems (San Diego, CA). Wheat germ–derived translation mixtures were depleted of Hsc70 by passage through the antibody column. The removal of Hsc70 was monitored by SDS-PAGE and protein gel blotting. Typically, three passes through the antibody column were necessary to completely remove the Hsc70.

### Analysis of in Vitro Translation Products

The association between the secretory protein precursor and Hsc70 was analyzed by rate-zonal sedimentation through a linear sucrose density gradient, essentially as described by Lecker et al. (1989). Briefly, samples (translation mixtures or standard proteins) were diluted with 10% sucrose, then loaded onto 12-mL linear 10 to 30% sucrose gradients containing 50 mM Tes-NaOH, pH 7.5, 35 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT. Gradients were formed using a BioComp Gradient Master and were centrifuged for 20 hr at 4°C and 36500 rpm in a Beckman SW-41 rotor using a Beckman L8-70M preparative ultracentrifuge. Gradients were analyzed as described by Riedell and Miernyk (1988). Maxidenz (Accurate Chemical and Scientific Company, Westbury, NY) was pumped into the bottom of the tubes using an Isco Model 185 Density Gradient Fractionator at a flow rate of 0.75 mL/min, and samples were collected by upward displacement. Samples of each fraction were removed to determine the sucrose concentration; an equal volume of 20% (w/v) trichloroacetic acid (TCA) was then added to the remainder. The fractions were kept on ice for at least 1 hr; the TCA-insoluble material was then collected by centrifugation at 4000 rpm for 20 min using a Beckman AccuSpin FR benchtop preparative centrifuge. The TCA-insoluble pellets were washed by resuspension in ice-cold 10% (w/v) TCA and recentrifugation, followed by resuspension in −20° acetone and recentrifugation. The final washed pellets were processed for analysis by SDS-PAGE and radioanalytic imaging.

### Immunoprecipitation of a Secretory Protein Precursor

The 50-μL translation mixtures were diluted to 0.5 mL with antibody buffer (50 mM Tes-NaOH, pH 7.5, containing 150 mM KCl, 5 mM EDTA, 2.5% [w/v] nonfat dry milk, and 0.05% [v/v] Nonidet P-40). After dilution, 20 μL of antiserum was added, and the samples were incubated for 12 hr on an orbiting rotator, followed by the addition of 20 μL of IgGsorb (The Enzyme Center, Inc., Malden, MA) and a further 4 hr of incubation. Samples were centrifuged at 14000 rpm and 4°C for 10 min using an Eppendorf 5414 centrifuge. The immunoprecipitates were washed by resuspension using a Kontes microcentrifuge pellet pestle, followed by recentrifugation. The immunoprecipitates were washed twice with PBS at room temperature and then washed five times with PBS containing 0.5% (v/v) Tween 20, 0.25% (v/v) Nonidet P-40, and 0.25% (w/v) Brij 35. The washed immunoprecipitates were then processed for SDS-PAGE and radioanalytic imaging (Shatters and Miernyk, 1991). When the temperature dependence of complex formation was to be studied, both immunoprecipitation and washing were conducted as indicated in the legend to Figure 3.

In some instances the effects of ATP on formation of the complex between pre-MRKΔXhoI and Hsc70 were tested directly. After dilution with antibody buffer, samples were divided into aliquots that were treated either by adding MgATP to a final concentration of 3 mM or by enzymatically removing ATP. Enzymatic removal was accomplished either by adding 20 units of apyrase or 10 mM glucose plus 5 units of hexokinase. Samples were then incubated at room temperature for 30 min before addition of antiserum.

### Other Analytical Methods

The methods for sample preparation, SDS-PAGE, protein staining, and protein gel blotting have been described (Miernyk, 1987; Hekman et al., 1990). Protein was quantitated by the method of Bradford (1976) using Fraction V BSA as the standard. Sucrose concentrations were determined using a refractometer. All sucrose concentrations presented are percent (w/w). All analyses, including quantitation of radioactive samples separated by SDS-PAGE, were conducted as described by Shatters and Miernyk (1991), using an AMBIS Radioanalytic Imaging System. Densitometry of Coomassie Brilliant Blue–stained gels and protein gel blots was accomplished with an LKB Ultrascan XL Enhanced Laser Densitometer. For phospho-amino acid analysis, samples were precipitated and washed three times with 10 volumes of ice-cold acetone, air-dried, and then acid hydrolyzed, and the hydrolysate was examined by two-dimensional thin layer electrophoresis (Huganir et al., 1984). The positions of phospho-amino acid standards were detected by spraying the plates with 0.25% (w/v) ninhydrin in acetone. Radioactive spots were detected by radioanalytic imaging.

## ACKNOWLEDGMENTS

We thank Stacey Sturch for general technical assistance; Nancy David for preparing the pea chloroplasts used in purification of the Cpn60 protein and the γ-$^{32}$P-ATP; and Frank Greene, Rebecca Boston, and Steve Gould for constructive criticism of the manuscript.

Received March 19, 1992; accepted May 19, 1992.

## REFERENCES

**Beckmann, R.P., Mizzen, L.A., and Welch, W.J.** (1990). Interaction of hsp70 with newly synthesized proteins: Implications for protein folding and assembly. Science **248,** 850–853.

**Boston, R.S., Fontes, E.B.P., Shank, B.B., and Wrobel, R.L.** (1991). Increased expression of the maize immunoglobulin binding protein homolog b-70 in three zein regulatory mutants. Plant Cell **3,** 497–505.

**Bradford, M.M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

**Chirico, W.J., Waters, M.G., and Blobel, G.** (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. Nature **332,** 805–810.

**Deshaies, R.J., Kock, B.D., Werner-Washburne, M., Craig, E.A., and Schekman, R.** (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. Nature **332,** 800–805.

**Earl, P.L., Moss, B., and Doms, R.W.** (1991). Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. **65,** 2047–2055.

**Ellis, R.J., and Hemmingsen, S.M.** (1989). Molecular chaperones: *Proteins essential for the biogenesis of some macromolecular structures.* Trends Biochem. Sci. **14,** 339–342.

**Ellis, R.J., van der Vies, S.M., and Hemmingsen, S.M.** (1989). The molecular chaperone concept. Biochem. Soc. Symp. **55,** 145–153.

**Garland, W.J., and Dennis, D.T.** (1980). Plastid and cytosolic phosphofructokinases from the developing endosperm of *Ricinus communis.* II. Comparison of the kinetic and regulatory properties of the isoenzymes. Arch. Biochem. Biophys. **204,** 310–317.

**Gatenby, A.A., and Ellis, R.J.** (1990). Chaperone function: The assembly of ribulose bisphosphate carboxylase-oxygenase. Annu. Rev. Cell Biol. **6,** 125–149.

**Grimm, R., Speth, V., Gatenby, A.A., and Schafer, E.** (1991). GroEL-related molecular chaperones are present in the cytosol of oat cells. FEBS Lett. **286,** 155–158.

**Hekman, W.E., Dennis, D.T., and Miernyk, J.A.** (1990). Secretion of *Ricinus communis* glyceraldehyde-3-phosphate dehydrogenase by *Escherichia coli.* Mol. Microbiol. **4,** 1363–1369.

**Hemmingsen, S.M.** (1990). The plastid chaperonin. Semin. Cell Biol. **1,** 47–54.

**Hemmingsen, S.M., and Ellis, R.J.** (1986). Purification and properties of ribulosebisphosphate carboxylase large subunit binding protein. Plant Physiol. **80,** 269–276.

**Hemmingsen, S.M., Woolford, C., van der Vies, S.M., Tilly, K., Dennis, D.T., Georgopoulos, C.P., Hendrix, R.W., and Ellis, R.J.** (1988). Homologous plant and bacterial proteins chaperone oligomeric protein assembly. Nature **333,** 330–334.

**Huganir, R.L., Miles, K., and Greengard, P.** (1984). Phosphorylation of the nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase. Proc. Natl. Acad. Sci. USA **81,** 6968–6972.

**Kumamoto, C.A.** (1991). Molecular chaperones and protein translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **5,** 19–22.

**Lecker, S., Lill, R., Ziegelhoffer, T., Georgopoulos, C., Bassford, P.J., Jr., Kumamoto, C.A., and Wickner, W.** (1989). Three pure chaperone proteins of *Escherichia coli*—SecB, trigger factor and GroEL—form soluble complexes with precursor proteins in vitro. EMBO J. **8,** 2703–2709.

**Leustek, T., Dalie, B., Amir-Shapira, D., Brot, N., and Weissbach, H.** (1989). A member of the Hsp70 family is localized in mitochondria and resembles *Escherichia coli* DnaK. Proc. Natl. Acad. Sci. USA **86,** 7805–7808.

**Lin, T-Y., Duck, N.B., Winter, J., and Folk, W.R.** (1991). Sequences of two hsc70 cDNAs from *Lycopersicon esculentum.* Plant Mol. Biol. **16,** 475–478.

**Lubben, T.H., Donaldson, G.K., Viitanen, P.V., and Gatenby, A.A.** (1989). Several proteins imported into chloroplasts form stable complexes with the GroEL-related chloroplast molecular chaperone. Plant Cell **1,** 1223–1230.

**Machamer, C.E., Doms, R.W., Bole, D.G., Helenius, A., and Rose, J.K.** (1990). Heavy chain binding protein recognizes incompletely disulfide-bonded forms of vesicular stomatitus virus G protein. J. Biol. Chem. **265,** 6879–6883.

**McCarty, J.S., and Walker, G.C.** (1991). DnaK as a thermometer: Threonine-199 is site of autophosphorylation and is critical for ATPase activity. Proc. Natl. Acad. Sci. USA **88,** 9513–9517.

**Miernyk, J.A.** (1987). Extracellular secretion of acid hydrolases by maize endosperm cells grown in liquid medium. J. Plant Physiol. **129,** 19–32.

**Miernyk, J.A., and Sturch, S.M.** (1991). Starvation for reduced nitrogen stimulates the secretion of proteases by maize endosperm cultures. J. Plant Physiol. **138,** 211–215.

**Mizzen, L.A., Kabiling, A.N., and Welch, W.J.** (1991). The two mammalian mitochondrial stress proteins, grp 75 and hsp 58, transiently interact with newly synthesized mitochondrial proteins. Cell Regul. **2,** 165–179.

**Paver, J.L., Hawkins, H.C., and Freedman, R.B.** (1989). Preparation and characterization of dog pancreas microsomal membranes specifically depleted of protein disulfide-isomerase. Biochem. J. **257,** 657–663.

**Pelham, H.R.B.** (1989). Heat shock and the sorting of luminal ER proteins. EMBO J. **8,** 3171–3176.

**Perdew, G.H., and Whitelaw, M.L.** (1991). Evidence that the 90-kDa heat shock protein (HSP90) exists in cytosol in heteromeric complexes containing HSP70 and three other proteins with $M_r$ of 63,000, 56,000, and 50,000. J. Biol. Chem. **266,** 6708–6713.

**Phillips, G.J., and Silhavy, T.J.** (1990). Heat-shock proteins DnaK and GroEL facilitate export of LacZ hybrid proteins in *E. coli.* Nature. **344,** 882–884.

**Riedell, W.E., and Miernyk, J.A.** (1988). Glycoprotein synthesis in maize endosperm cells. The nucleoside diphosphate-sugar: Dolichol-phosphate glycosyltransferases. Plant Physiol. **87,** 420–426.

**Rochester, D.E., Winter, J.A., and Shah, D.M.** (1986). The structure and expression of maize genes encoding the major heat shock protein, hsp70. EMBO J. **5,** 451–458.

**Rothman, J.E.** (1989). Polypeptide chain binding proteins: Catalysts of protein folding and related processes in cells. Cell **59,** 591–601.

**Shatters, R.G., Jr., and Miernyk, J.A.** (1991). A zein signal sequence functions as a signal-anchor when fused to maize alcohol dehydrogenase. Biochim. Biophys. Acta **1068,** 179–188.

**Vierling, E.** (1991). The roles of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42,** 579–620.

**Vogel, J.P., Misra, L.M., and Rose, M.D.** (1990). Loss of BiP/grp78 function blocks translocation of secretory proteins in yeast. J. Cell Biol. **110,** 1885–1895.

**Walker, J.C., and Zhang, R.** (1990). Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica.* Nature **345,** 743–746.

**Welch, W.J., and Feramisco, J.R.** (1985). Rapid purification of mammalian 70,000-dalton stress proteins: Affinity of the proteins for nucleotides. Mol. Cell. Biol. **5,** 1229–1237.

**Zimmermann, R., Sagstetter, M., Lewis, M.J., and Pelham, H.R.B.** (1988). Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. EMBO J. **7,** 2875–2880.

**Zylicz, M., LeBowitz, J.H., McMacken, R., and Georgopoulos, C.** (1983). The dnaK protein of *Escherichia coli* possesses an ATPase and autophosphorylating activity and is essential in an in vitro DNA replication system. Proc. Natl. Acad. Sci. USA **80,** 6431–6435.