

1997

Complexes Between Nascent Polypeptides and Their Molecular Chaperones In the Cytosol of Mammalian Cells

Daryl K. Eggers, *San Jose State University*

W. J Welch, *University of California, San Francisco*

W. J Hansen, *University of California, San Francisco*

Complexes between Nascent Polypeptides and Their Molecular Chaperones in the Cytosol of Mammalian Cells

Daryl K. Eggers, William J. Welch,* and William J. Hansen

Department of Cellular and Molecular Pharmacology and Departments of Medicine and Physiology,
University of California, San Francisco, San Francisco, California 94143

Submitted March 14, 1997; Accepted May 20, 1997

Monitoring Editor: David G. Drubin

Folding of newly synthesized proteins *in vivo* is believed to be facilitated by the cooperative interaction of a defined group of proteins known as molecular chaperones. We investigated the direct interaction of chaperones with nascent polypeptides in the cytosol of mammalian cells by multiple methods. A new approach using a polyclonal antibody to puromycin allowed us to tag and capture a population of truncated nascent polypeptides with no bias as to the identity of the bound chaperones. In addition, antibodies that recognize the cytosolic chaperones hsp70, CCT (TRiC), hsp40, p48 (Hip), and hsp90 were compared on the basis of their ability to coprecipitate nascent polypeptides, both before and after chemical cross-linking. By all three approaches, hsp70 was found to be the predominant chaperone bound to nascent polypeptides. The interaction between hsp70 and nascent polypeptides is apparently dynamic under physiological conditions but can be stabilized by depletion of ATP or by cross-linking. The cytosolic chaperonin CCT was found to bind primarily to full-length, newly synthesized actin, and tubulin. We demonstrate and caution that nascent polypeptides have a propensity for binding many proteins nonspecifically in cell lysates. Although current models of protein folding *in vivo* have described additional components in contact with nascent polypeptides, our data indicate that the hsp70 and, perhaps, the hsp90 families are the predominant classes of molecular chaperones that interact with the general population of cytosolic nascent polypeptides.

INTRODUCTION

It is well known that the primary sequence of amino acids contains all the information necessary for protein folding (Anfinsen, 1973). Inside the cell, however, the pathway of protein folding is much different from that observed for the refolding of chemically or thermally denatured proteins. During the early stages of protein synthesis, a nascent polypeptide has not yet acquired all of the necessary information (i.e., a suitable number of amino acids) to carry out proper folding. In addition, the nascent polypeptide must contend with a crowded environment where incidental binding between the nascent polypeptide and its nearest neighbors could lead conceivably to nonproduc-

tive folding or aggregation. A class of proteins, now referred to as molecular chaperones, is believed to help prevent such nonproductive interactions by shielding or stabilizing critical intermediates during the folding process (reviewed by Gething and Sambrook, 1992; Georgopoulos and Welch, 1993; Becker and Craig, 1994; Hendrick and Hartl, 1995; Buchner, 1996).

The two main classes of molecular chaperones implicated in the folding of nascent or newly synthesized polypeptides are the hsp60 and hsp70 families. Members of both families are highly conserved, and the expression of some (but not all) members is increased after different types of metabolic stress, including heat shock. Because metabolic stress often results in destabilization and aggregation of proteins, molecular chaperones are thought to play an important role in

* Corresponding author.

the renaturation or prevention of such aggregates, in addition to their role in facilitating the folding of newly synthesized proteins. The latest concepts on the mechanisms of action of the hsp60 and the hsp70 chaperone families recently have been reviewed (Hartl, 1996). Members of both families hydrolyze ATP in a reaction cycle that alters their conformation and thereby modulates their affinity for unfolded model substrates. The ATP reaction cycle for hsp70 (and its bacterial homologue DnaK) may be mediated by other proteins in vivo including hsp40 (DnaJ in bacteria), p48 (also known as Hip), and GrpE (identified so far in bacteria and mitochondria only).

On the basis of chaperone interactions observed in a number of different systems including protein transport into isolated mitochondria, refolding experiments with purified bacterial chaperones, and in vitro translation experiments, a model of protein folding in the eukaryotic cytosol has been proposed (Frydman *et al.*, 1994; Frydman and Hartl, 1996). Briefly, Hartl (1996) has suggested that nascent polypeptides are first recognized by and bound to hsp40 (the hsp40 gene product). Binding of hsp40 then acts to recruit cytosolic hsp70 and thereby facilitate its interaction with the nascent polypeptide. For some proteins, a subsequent interaction with the cytosolic chaperonin would then represent the last step in the protein maturation process (Hartl, 1996). Support for different aspects of this proposed pathway has been provided by many laboratories. Members of the hsp70 family have been reported to coprecipitate newly synthesized proteins in mammalian cells (Beckmann *et al.*, 1990), to cosediment with nascent polypeptides still bound to polysomes (Nelson *et al.*, 1992; Beck and De Maio, 1994), and to coprecipitate truncated forms of nascent polypeptides after translation in vitro (Frydman *et al.*, 1994; Hansen *et al.*, 1994; Frydman and Hartl, 1996), results that all implicate this chaperone in the early events of protein folding. The cytosolic chaperonin containing TCP-1, CCT (also referred to as TRiC), is distantly related to the hsp60 family and forms a large heteromeric ring complex (Kubota *et al.*, 1995). CCT has been reported to bind newly synthesized tubulin and actin (Sternlicht *et al.*, 1993; Dobrzynski *et al.*, 1996), to participate in assembly of viral capsids (Lingappa *et al.*, 1994), and to bind nascent forms of both firefly luciferase and actin (Frydman *et al.*, 1994; Frydman and Hartl, 1996). Hsp40 and p48 also have been reported to bind truncated forms of firefly luciferase, suggesting that these proteins may have a chaperone function in addition to their roles as cofactors for the ATP-hydrolysis cycle of hsp70 (Frydman *et al.*, 1994; Höhfeld *et al.*, 1995).

Experimental approaches for studying protein folding have become more sophisticated to simulate the folding process in vivo. For example, refolding experiments have been performed in the presence of whole

cell lysates (Schumacher *et al.*, 1994; Tian *et al.*, 1996) or purified molecular chaperones (Langer *et al.*, 1992; Schröder *et al.*, 1993; Szabo *et al.*, 1994; Jakob *et al.*, 1995; Freeman and Morimoto, 1996), and in vitro translation systems have been used to identify proteins that interact with ribosome-bound nascent polypeptides (Hendrick *et al.*, 1993; Kudlicki *et al.*, 1994; Wiedmann *et al.*, 1994; Hesterkamp *et al.*, 1996; Reid and Flynn, 1996). All of these approaches are dependent on the selection of a model protein, and, therefore, the chaperone interactions that are detected in these systems do not apply necessarily to the folding of all proteins. Here we have used a number of different approaches to examine the general population of nascent polypeptides and their associated molecular chaperones in the cytosol of mammalian cells. Immunoprecipitations with antibodies to a selected set of putative molecular chaperones have been carried out to determine which of these proteins interact directly with nascent polypeptides. For some experiments, chemical cross-linking was performed in vivo to stabilize the nascent chains with their associated chaperones before cell lysis and immunoprecipitation analysis. In addition, we have continued to exploit the use of an antibody to puromycin in our studies, as first described in a previous communication (Hansen *et al.*, 1994). Puromycin is an antibiotic that competes with aminoacyl-tRNAs for the A-site of ribosomes. Once bound to the ribosome, puromycin becomes covalently incorporated into the C terminus of the polypeptide chain resulting in premature termination. This property of puromycin is used to "tag" nascent polypeptides synthesized in vivo and to selectively isolate the polypeptides with the polyclonal antibody to puromycin. At low concentrations of puromycin, it is possible to accumulate a large population of nascent polypeptides derived from all the actively translated mRNAs in the cell. This heterogeneous collection of "puromycyl-polypeptides" may yield information about molecular chaperone interactions that is more generally characteristic of the folding and maturation of cytosolic proteins.

MATERIALS AND METHODS

Metabolic Labeling, Puromycin Treatment, and Cell Lysis

HeLa cells were grown in 10% calf serum/DMEM as monolayers on culture plates to 50–75% confluency. Typically, 250–500 μ Ci of [35 S]methionine (Dupont-New England Nuclear NEG-072, 1000 Ci/mmol) in 5 ml of medium was used for metabolic labeling of a 10-cm plate of cells. Pulse labeling was performed in methionine-free DMEM for short times as indicated in the figure legends, and the cells were lysed immediately without a chase period. Steady-state labeling was achieved by incubating the cells with label overnight (14–18 h) in methionine-free DMEM supplemented with 5% complete DMEM and 2% calf serum. Overnight labeling was followed by a minimum 4-h chase period in complete medium containing 10% calf serum (no label) to ensure that all labeled proteins

were given time to mature before treatment with puromycin and cell lysis. Unless noted otherwise, puromycin (Sigma Chemical Co., St. Louis, MO) was added to the medium at a concentration of 2 μ M for 20 min just before cell lysis. For pulse-labeling experiments, puromycin and [35S]methionine often were added simultaneously. See figure legends for exact duration of pulse labeling and puromycin treatments. Cells were washed with phosphate-buffered saline (PBS) and placed at 4°C before being harvested by scraping into lysis buffer. Typically, 1 ml of buffer A (20 mM HEPES, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol) containing 0.1% Triton X-100 was used per 10-cm plate for detergent lysis. For hypotonic lysis, the cells were incubated at 4°C in 10 mM HEPES, 10 mM KCl, and 0.1 mM EDTA and allowed to swell at least 15 min while still on the plate. The cells then were scraped off the plate, and the lysate was adjusted with salts to achieve final buffer A concentrations. Lysates were extruded through a 26-gauge needle, incubated for 10 min at 4°C with 10 U/ml apyrase (Sigma) to deplete ATP, and clarified in a microcentrifuge for 10 min. In some experiments, the lysates were supplemented with Mg-ATP in place of the apyrase treatment (see figure legends). We have found that commercial apyrase preparations occasionally are contaminated with a protease activity that can be reduced by preincubation with soybean trypsin inhibitor at a concentration of 200 ng/unit apyrase. For the experiments in Figure 8, bovine serum albumin (BSA), reduced carboxymethylated-BSA, and α -casein (all from Sigma) were added to buffer A + 0.1% Triton X-100 at a concentration of 10 mg/ml before cell lysis. For the proteolysis experiment of Figure 3, TPCK-treated trypsin was used (Worthington Biochemical, Freehold, NJ).

Antibodies and Immunoprecipitation Techniques

For immunoprecipitation under denaturing conditions, cell lysates were adjusted with Laemmli sample buffer (final, 1% SDS) and heated at 95°C for 5 to 10 min. After clarification in a microcentrifuge, the lysate was diluted in RIPA buffer (1% Triton X-100, 1% deoxycholate in PBS) such that the final concentration of SDS was always less than 0.05%. All samples were preadsorbed with Sepharose CL-4B (Pharmacia, Piscataway, NJ) for 30 min before addition of antibody. Incubations with the primary antibody were carried out for a minimum of 2 h at 4°C, followed by a 1-h incubation with rabbit anti-mouse and rabbit anti-rat secondary antibodies, when appropriate. The resulting complexes were captured with protein A Sepharose CL-4B (Pharmacia) and washed four times with RIPA buffer supplemented with 0.1% SDS.

For examining protein-protein interactions, immunoprecipitation under "native" or "nondenaturing conditions" was performed. In this case, the cells were both lysed and diluted in buffer A + 0.1% Triton X-100 at 4°C (no SDS, no heating). Antibody additions and immunoprecipitations were done as described above, except that the final immunoprecipitates were washed four times with buffer A supplemented with 1% Triton X-100 and 1% sodium deoxycholate.

The polyclonal antibody to puromycin was developed by conjugating the molecule to a mixture of keyhole limpet hemocyanin and hen egg white lysozyme and combining with complete Freund's adjuvant before injection of rabbits. A mixture of antibodies was used for precipitation of hsc70/hsp70, which included four mouse monoclonal antibodies (N6, N15, N21, N33; Milarski *et al.*, 1989), a rabbit peptide antibody to hsc70 (Brown *et al.*, 1993), and a rat monoclonal antibody 1B5 (Stressgen). The mouse monoclonal antibody N27 (Stressgen Biotech Corp., Victoria, British Columbia, Canada) was added to the hsp70 antibody mixture for analysis of denatured samples. A mixture of mouse antibodies was used to capture p48 (2G6, 1A6, 10D1; Prapapanich *et al.*, 1996). The P5 antibody that recognizes the γ -subunit of CCT was used for non-denatured samples only (Joly *et al.*, 1994). For immunoprecipitation of CCT from denatured samples, a mixture of a rabbit polyclonal antibody made against a synthetic peptide of mouse TCP-1 (Lingappa *et al.*, 1994) and the rat monoclonal antibodies 84a and 91a (Willison *et al.*, 1989, obtained from Stressgen) was used. A rabbit

polyclonal antibody to hsp40 was prepared using recombinant human hsp40 as immunogen (Nagata *et al.*, manuscript in preparation). A rabbit polyclonal antibody to hsp90 was used for immunoprecipitation of nondenatured samples (W.J.W., unpublished observations) and was supplemented with the rat monoclonal antibodies 16F1 and 9D2 (obtained from Stressgen; Lai *et al.*, 1984) for the analysis of denatured samples.

Sizing Chromatography and Sedimentation Analyses

Sizing chromatography was performed in a 1.0 \times 20-cm interior diameter column packed with Sephacryl S-400 (Sigma), which was equilibrated in buffer A. The excluded volume of the column was measured by the elution of phage λ DNA, followed by ethidium bromide staining. The sedimentation profile of puromycyl-polypeptides was achieved by layering a puromycin-treated lysate on a 9-ml linear gradient of 10–40% sucrose in buffer A + 0.1% Triton X-100 with a 1-ml bottom cushion of 60% sucrose in the same buffer. Centrifugation was carried out in a SW-41 rotor (Beckman) for 24 h at 39,000 rpm and 4°C. Fractions were collected and analyzed by immunoprecipitation as described in the figure legend. Fractions containing the 20 s proteasome were determined by immunoprecipitation using a polyclonal antibody against one of the proteasome subunits (kindly provided by A.L. Goldberg, Harvard University, Cambridge, MA). Other sedimentation markers (indicated in Figure 2) were added directly to the samples and visualized by Coomassie stain after SDS-PAGE. For isolation of polysomes, cells were treated with 0.1 mg/ml of cycloheximide before hypotonic lysis and layered on top of 5 ml of 20% sucrose in buffer A + cycloheximide with a 2-ml cushion of 70% sucrose in the same buffer. Polysomes were found near the interface of the two sucrose layers after a 2-h centrifugation at 39,000 rpm.

Chemical Cross-Linking In Vivo

After labeling with [35S]methionine for the times indicated in Figure 5, HeLa cells were treated with 0.1 mg/ml of cycloheximide for 2 min at 37°C to stabilize polysomes. The cells were rinsed with PBS containing 0.1 mg/ml cycloheximide and incubated for 10 min at ambient temperature (20–22°C) in the same solution supplemented with 2 mM DSP, the cell permeable cross-linker dithiobis(succinimidyl-propionate), from Pierce Chemicals (Rockford, IL) (Safiejko-Mroccka and Bell, 1996). After removal of the DSP solution, the reaction was quenched by incubating the cells in 50 mM glycine and 50 mM Tris, pH 7, for 10 min. The cells then were lysed in Laemmli sample buffer without any reducing agent and heated for 10 min at 75°C. Immunoprecipitation under denaturing conditions was performed using antibodies against the different molecular chaperones as given in the figure legends. The final immunoprecipitates were boiled in Laemmli sample buffer containing extra reducing agent (0.25 M dithiothreitol and 0.25 M β -mercaptoethanol) to reverse the cross-links.

Electrophoresis Techniques

Protein samples were analyzed by SDS-PAGE and fluorography on gels containing a constant product of acrylamide and bisacrylamide (%A \times %B = 1.3; Blattler *et al.*, 1972). The isoelectric gradient for two-dimensional gels was developed using ampholines of 70% pH 5–7 and 30% pH 3–10 (Pharmacia). A 12.5% acrylamide gel was used for the second dimension.

RESULTS

Isolation of Puromycyl-Polypeptides from Animal Cells

Addition of puromycin to growing cells at low concentrations resulted in an accumulation of nascent

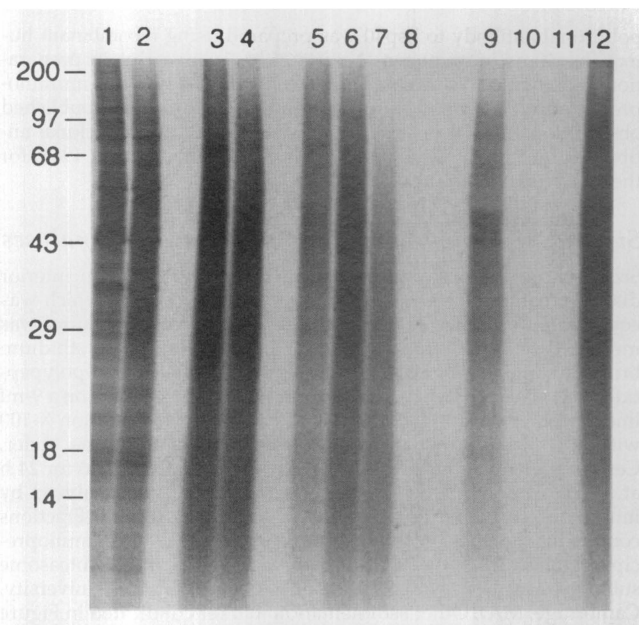


Figure 1. Characterization of the antipuumycin antibody. HeLa cells were labeled for 20 min with [35 S]methionine and incubated with puromycin as indicated below before cell lysis. Lanes 1–2 are starting lysates before immunoprecipitation; lanes 3–8, 10 and 11 are immunoprecipitations with the puromycin antibody under non-denaturing conditions. Lane 1, total lysate, no puromycin; lane 2, total lysate, 2 μ M puromycin for 30 min; lane 3, 2 μ M puromycin for 15 min; lane 4, 2 μ M puromycin for 30 min; lane 5, 0.4 μ M puromycin for 30 min; lane 6, 2 μ M puromycin for 30 min; lane 7, 10 μ M puromycin for 30 min; lane 8, 50 μ M puromycin for 30 min; lane 9, immunoprecipitation under denaturing conditions, 2 μ M puromycin for 30 min; lane 10, blocked antibody control, 100 μ M puromycin added to diluted lysate before antibody; lane 11, control, preimmune serum; lane 12, coprecipitation with antibodies to hsc70/hsp70 under non-denaturing conditions using fivefold more volume of the same lysate used in lane 6.

puromycyl-polypeptides without an immediate shutdown of protein synthesis. After a short labeling period with [35 S]methionine in the presence of puromycin, the puromycyl-polypeptides were isolated with a polyclonal antibody specific for puromycin. When analyzed by SDS-PAGE, the puromycin-released polypeptides appeared as a “smear” of radiolabeled proteins migrating throughout the gel (Figure 1). This heterogeneous appearance is what should be expected for a population of polypeptides that were derived from all of the active mRNAs in the cell by puromycin incorporation at random points in translation. Regardless of the brevity of the labeling period, a number of discrete bands always were observed against this background smear of puromycyl-polypeptides in the total cell lysates. These discrete bands represent full-length translation products that were terminated normally in the cells without incorporation of the antibiotic (Figure 1, lanes 1–2). Consistent with this interpretation is the fact that few, if any, full-length proteins were

immunoprecipitated using the antipuumycin antibody (Figure 1, lanes 3–9). Production of puromycyl-polypeptides showed little dependence on incubation times beyond 15 min when using a concentration of 2 μ M puromycin (Figure 1, lanes 3–4), but a strong dependence on puromycin concentration for a given incubation time (Figure 1, lanes 5–8). In general, 2 μ M puromycin for 15 to 30 min yielded the highest accumulation of released nascent polypeptides exhibiting a broad distribution of molecular sizes (Figure 1, lane 6). Concentrations of puromycin higher or lower than 2 μ M resulted in nascent polypeptides of either smaller or larger average molecular mass, respectively. No accumulation of nascent polypeptides was observed at puromycin concentrations above 50 μ M, as analyzed by 12.5% polyacrylamide gels (Figure 1, lane 8). Previous incubation of the serum with free puromycin effectively blocked the capture of puromycyl-polypeptides (Figure 1, lane 10). Consistent with previous observations, puromycin-released nascent chains that were captured by the antibody to puromycin were similar in size to those captured using antibodies to the cytosolic chaperones hsc70 and hsp70 (Beckmann *et al.*, 1990). Note that the constitutive hsc70 (also known as hsp73) and the highly stress-inducible hsp70 (hsp72) represent the two major cytosolic members of the family and will be referred to here collectively as “hsp70.” These results demonstrate the utility of puromycin and confirm the specificity of the puromycin antibody for the capture and characterization of nascent polypeptides.

Complexes between Nascent Polypeptides and Their Chaperones Are Large and Heterogeneous in Size

The antibody to puromycin was used to examine some of the physical characteristics of nascent polypeptides. Via both sizing chromatography and velocity sedimentation through a 10–40% sucrose gradient, the population of puromycin-released nascent polypeptides was observed to be heterogeneous in size. When a pulse-labeled puromycin-treated lysate was eluted from a Sephacryl S-400 sizing column, the majority of the puromycyl-polypeptide chains fractionated with native molecular masses between the 66 and 670 kDa size markers (Figure 2A). Most of the puromycyl-polypeptides sedimented between 2 and 11 s after velocity sedimentation of an identically prepared cell lysate (Figure 2B).

When steady-state labeled puromycin-treated cell lysates were fractionated by the same two methods, a number of mature proteins were observed to coprecipitate with the puromycin antibody (Figure 2, C and D). The binding of these proteins may contribute to the native size of the nascent polypeptide complexes

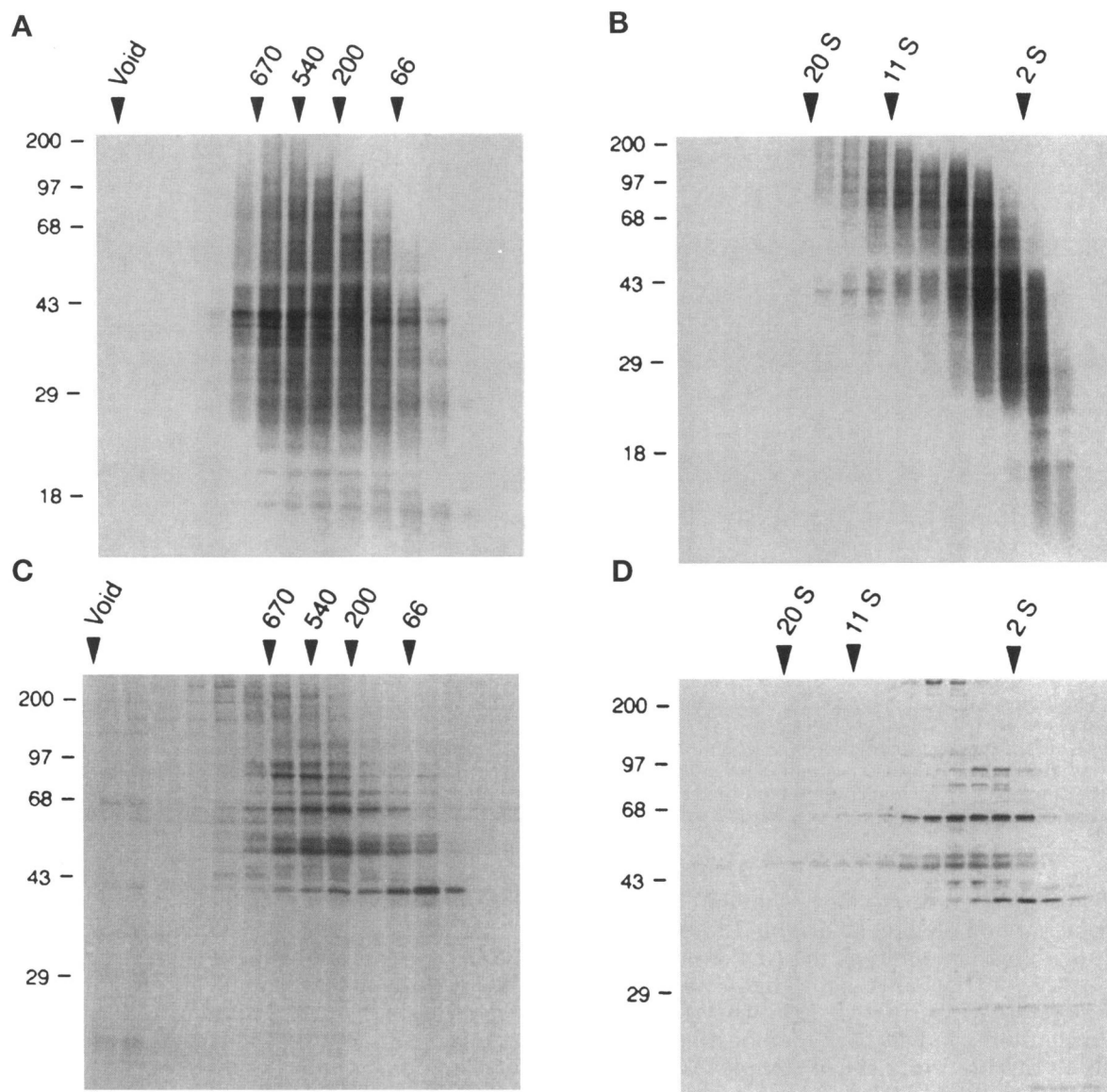


Figure 2. Native size of protein complexes with nascent puromycyl-polypeptides. (A and C) Sizing chromatography with an S-400 column. Elution is from left to right. Native size markers are thyroglobulin, 670 kDa; urease, 540 kDa; β -amylase, 200 kDa; and hemoglobin, 66 kDa. (B and D) Sedimentation analysis using a 10–40% sucrose gradient. The bottom of the gradient is at left. Sedimentation markers are the 20 s proteasome particle; catalase, 11 s; and cytochrome c, 2 s. See MATERIALS AND METHODS for details of separation by chromatography and velocity sedimentation. For A and B, starting lysates were obtained from HeLa cells that had been labeled with [35 S]methionine for 20 min in the simultaneous presence of 2 μ M puromycin. Fractions containing the pulse-labeled proteins were immunoprecipitated with the puromycin antibody under denaturing conditions before analysis on 12.5% acrylamide gels. For C and D, starting lysates were obtained from cells that had been steady-state labeled with [35 S]methionine and chased for 4 h before a 20-min incubation with 2 μ M puromycin. Fractions containing steady-state labeled proteins were immunoprecipitated with the puromycin antibody under nondenaturing conditions before analysis on 10% acrylamide gels.

observed in Figure 2, A and B. The molecular masses of the individual proteins that coprecipitated with the puromycyl-polypeptides were similar after both chromatography and sedimentation analyses. Some of these steady-state labeled proteins will be identified below.

Nascent Polypeptides Are More Sensitive to Proteases in the Presence of Mg-ATP

Members of the hsp60 and hsp70 chaperone families are known to have ATPase activities that modulate their conformation and affinity for bound substrates

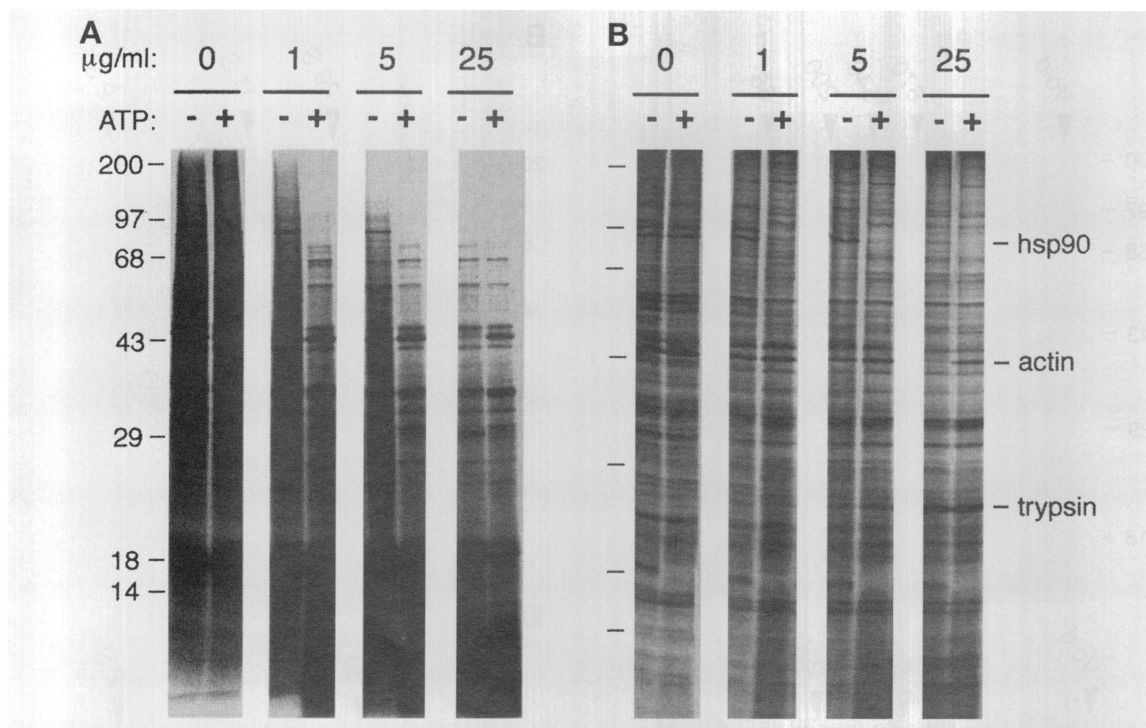


Figure 3. Sensitivity of nascent polypeptides (A) and mature proteins (B) to degradation by exogenous protease in the absence or presence of ATP. Two plates of HeLa cells were labeled with [35 S]methionine for the last 10 min of a 20-min incubation with 2 μ M puromycin. One plate was lysed in the presence of apyrase (–ATP), and the other plate was lysed with 5 mM MgATP in the buffer (+ATP). A stock solution of trypsin was added at the indicated concentrations to 20- μ l aliquots of lysate and incubated for 30 min at 22°C. Proteolysis was terminated by boiling the samples in 3 \times Laemmli sample buffer and analyzed in duplicate on 12.5% acrylamide gels. (A) Autoradiograph. (B) Gel stained with silver reagent.

(Hartl, 1996). Previous studies have shown that hsp70 and its bacterial homologue, DnaK, bind to unfolded substrates with higher affinity in the ADP-bound state (Palleros *et al.*, 1991; Sadis and Hightower, 1992; Schmid *et al.*, 1994; McCarty *et al.*, 1995; Theyssen *et al.*, 1996). Furthermore, it has been demonstrated that hsp70 can be captured in a stable complex with nascent polypeptides after ATP levels have been depleted in a HeLa cell lysate (Beckmann *et al.*, 1990). To study the environment of the nascent polypeptides as a function of ATP, we examined the accessibility of the nascent chains to an added protease. Using trypsin digestion as a probe of nascent chain accessibility in pulse-labeled cell lysates, proteolysis showed a clear dependence on ATP levels (Figure 3A). Specifically, in those lysates where ATP had been depleted, the overall extent of proteolytic digestion of the radiolabeled nascent chains was significantly less as compared with those lysates where ATP had been added. Note that the discrete bands representing full-length radiolabeled proteins were not degraded at any of the trypsin concentrations used (Figure 3A). Instead, the primary target of the added protease was the smear of radioactive material that represents the nascent polypeptides.

Examination of the proteins in the same cell lysates by SDS-PAGE and silver staining revealed that the protease concentrations that were used did not result in global protein degradation (Figure 3B). Very few of the mature silver-stained proteins were affected by the added protease. It was of interest that hsp90 exhibited enhanced degradation in those lysates supplemented with ATP. On the other hand, actin degradation was observed to increase after ATP depletion. A similar pattern of degradation was obtained with cells that were treated with cycloheximide (Eggers, 1997). These results are consistent with the idea that many of the nascent chains form complexes with other macromolecules and that some of these “protective macromolecules” represent members of the ATP-dependent molecular chaperone families.

The Predominant Molecular Chaperone Associated with Nascent Puromycyl-Polypeptides Is hsp70

To identify components interacting with nascent polypeptides, HeLa cells were steady state labeled with [35 S]methionine before puromycin treatment. The (unlabeled) puromycyl-polypeptides were captured using the puromycin antibody, and the (radio-

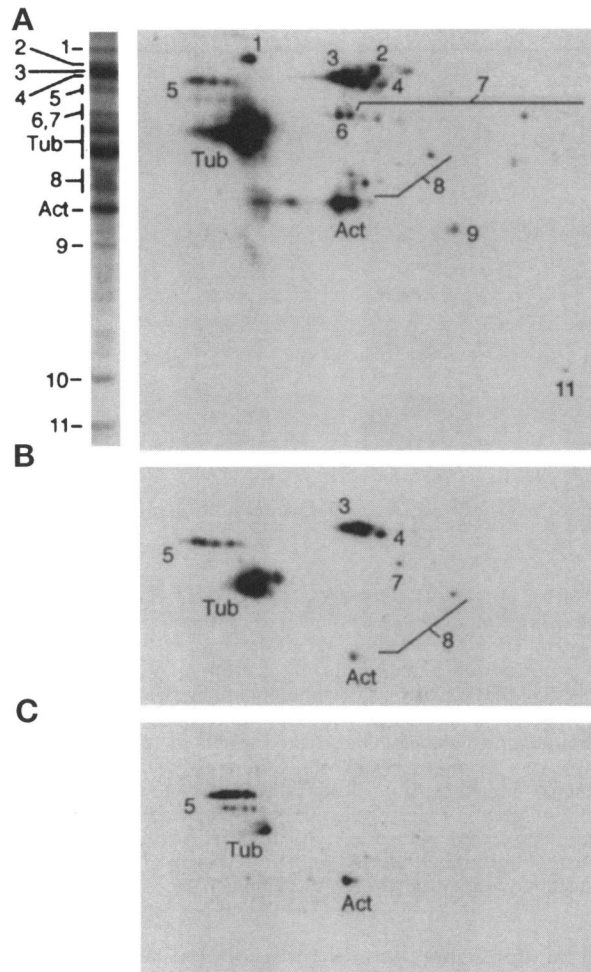


Figure 4. Identification of proteins that coprecipitate with puromycin-polypeptides by two-dimensional gel analysis. (A) HeLa cells were labeled with [35 S]methionine overnight, chased for 4 h, and treated for 30 min with 2 μ M puromycin before detergent lysis and immunoprecipitation with the antipuromycin antibody under non-denaturing conditions. A one-dimensional profile of the same sample from another gel is shown at left. (B) Mature proteins were labeled and treated with puromycin as in (A), but the cells were lysed hypotonically to remove the organelles before immunoprecipitation with the antipuromycin antibody. (C) Antipuromycin immunoprecipitation from hypotonically lysed cells that were not treated with puromycin. Proteins of more basic isoelectric points are seen on the right side of each two-dimensional gel. The identified proteins are as follows: (1) BiP; (2) grp75; (3) hsc70; (4) hsp70; (5) unknown; (6) hsp60; (7) CCT; (8) cyto keratins; (9–11) unknown; (Tub) α - and β -tubulin; (Act) actin.

labeled) mature proteins that coprecipitated with the nascent polypeptides were analyzed by both one and two-dimensional gel electrophoresis (Figure 4). The proteins that coprecipitated from the whole lysate had a similar pattern by SDS-PAGE to those proteins that were observed to cofractionate with puromycin-polypeptides after sizing chromatography and velocity sedimentation (compare the one-dimensional gel

profile of Figure 4A to Figure 2, C and D). The major coprecipitating proteins are numbered for reference (1–11). The identity of many of the coprecipitating proteins was determined by immunoprecipitation with protein-specific antibodies; the two-dimensional gel coordinates of known antigens were aligned with the coordinates of the proteins that coprecipitated with the puromycin-polypeptides (Eggers, 1997). The most prominent molecular chaperones that coprecipitated with the puromycin-polypeptides were members of the hsp70 family. In addition to the cytosolic forms of hsp70, i.e., hsc70 (3) and hsp70 (4), both the endoplasmic reticulum form of hsp70, BiP (1), and the mitochondrial form of hsp70, grp75 (2), were identified. Low levels of the mitochondrial chaperonin, hsp60 (6), also were detected. Note, however, that little of the heteromeric cytosolic chaperonin, CCT (7), was coprecipitated (a typical two-dimensional profile of CCT is shown in Figure 6B). The capture of the compartmentalized chaperones (BiP, grp75, and the mitochondrial hsp60) with the puromycin-released nascent chains no longer was observed when the cells were lysed via hypotonic treatment, followed by removal of the intact organelles by velocity sedimentation (Figure 4B). In this case, the major components that coprecipitated with the cytosol-enriched nascent chains were hsc70 and hsp70 (3 and 4). The unidentified species marked 5, 9, and 10 were bound to the puromycin antibody nonspecifically because they also were precipitated from lysates that had never been treated with puromycin (see Figure 4C for example of nonspecific binding by 5). Of the other proteins that coprecipitated with puromycin-polypeptides, many were identified as structural subunits of the cytoskeleton, including actin, tubulin, and some intermediate filament proteins (8). Conditions for reducing the binding of these cytoskeletal proteins are discussed later.

As a complementary approach to identify molecular chaperones that interact with nascent polypeptide chains, immunoprecipitation studies were carried out using antibodies specific for a selected set of molecular chaperones. The specificity of the various antibodies was confirmed by immunoprecipitation of steady-state labeled lysates under nondenaturing conditions (Figure 5A). A collection of antibodies against cytosolic hsp70 precipitated both the constitutive and inducible forms (Figure 5A, lane 1). An antibody to the γ -subunit of CCT resulted in the capture of a number of proteins of approximately 55–65 kDa size by SDS-PAGE, consistent with the heteromeric nature of the cytosolic chaperonin particle (Figure 5A, lane 2). A recently prepared antibody to hsp40 resulted in its isolation along with another prominent protein of approximately 65 kDa, the latter which may represent another DnaJ homologue present in animal cells (Nagata *et al.*, manuscript in preparation). Antibodies to

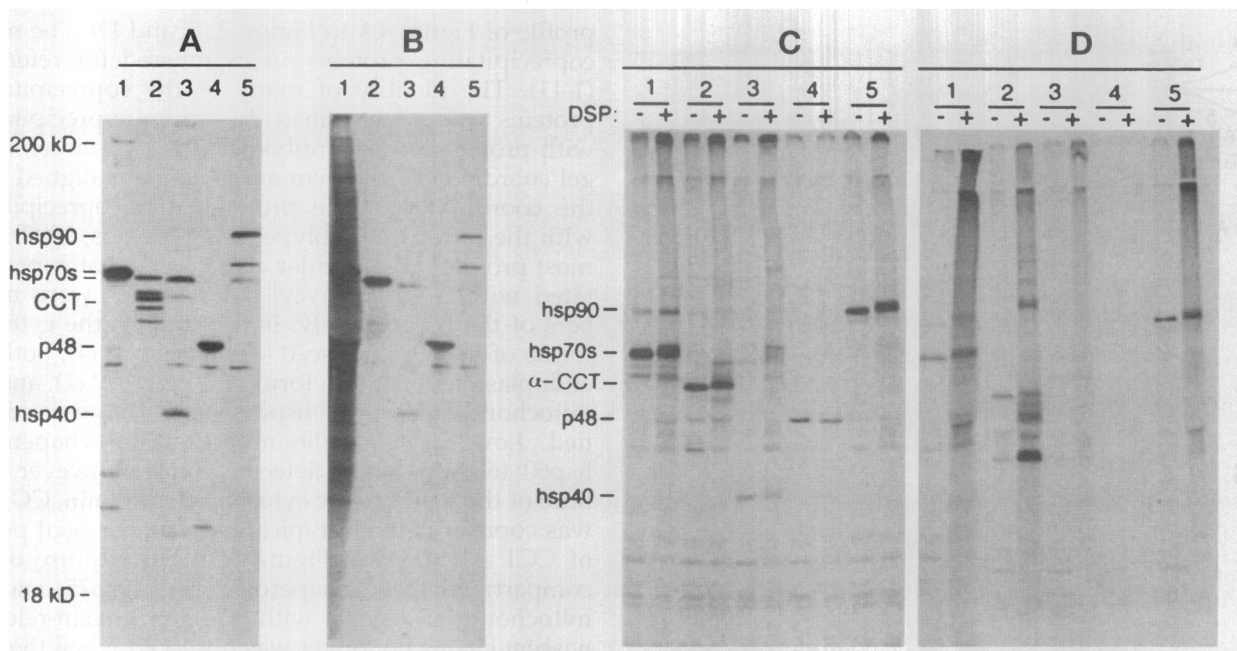


Figure 5. Ability of antichaperone antibodies to coprecipitate nascent polypeptides under nondenaturing condition (A and B) or under denaturing conditions after cross-linking in vivo (C and D). See MATERIALS AND METHODS for description of antibodies used and details of cross-linking experiment. (A) HeLa cells were steady-state labeled with [35 S]methionine, treated with 2 μ M puromycin for 20 min, and immunoprecipitated under nondenaturing conditions. (B) HeLa cells were simultaneously labeled and treated with 2 μ M puromycin for 20 min, followed by immunoprecipitation under nondenaturing conditions. (C) Two plates of HeLa cells were steady-state labeled with [35 S]methionine and treated with cycloheximide. One plate was used as an uncross-linked control (–), and the other plate was treated with 2 mM DSP (+) before immunoprecipitation under denaturing conditions. (D) Two plates of HeLa cells were pulse-labeled for 5 min before cross-linking and immunoprecipitation as in (C). In each panel, immunoprecipitations were performed with antibodies which recognize the following antigens: (1) hsc70/hsp70; (2) CCT; (3) hsp40; (4) p48; (5) hsp90. Note that the antibodies used for CCT and hsp90 in panels (C) and (D) were altered from panels (A) and (B) to include reagents that worked effectively under denaturing conditions.

p48/Hip brought down an approximately 48-kDa antigen, whereas the polyclonal hsp90 antibody precipitated hsp90 along with minor amounts of two other proteins. When we examined the relative interaction of these different molecular chaperones with pulse-labeled nascent polypeptides released in vivo via puromycin treatment, a significant amount of the puromycyl-polypeptides was observed to coprecipitate with cytosolic hsp70 (Figure 5B, lane 1). In contrast, antibodies to CCT, hsp40, p48, and hsp90, while capturing their respective full-length newly synthesized antigens, failed to coprecipitate the smear of puromycin-released nascent chains. Typically, coprecipitation of puromycyl-polypeptides with the pool of anti-hsp70 antibodies yielded 15–20% of the total polypeptides captured with the polyclonal antibody to puromycin.

To stabilize ribosome-bound nascent chains with their relevant molecular chaperones in the presence of physiological concentrations of nucleotides and total protein, a cell permeable cross-linker was used. First, it was confirmed that the different molecular chaperones could be captured by their respective antibodies after chemical cross-linking. Steady-state labeled cells were incubated with the membrane-permeable and thiol-reversible cross-linker DSP before cell lysis. As a

control, a second plate of radiolabeled cells was incubated under the same conditions without the cross-linking reagent, and both plates of cells were harvested under denaturing conditions such that only the cross-linked interactions should remain intact. Immunoprecipitation reactions using antibodies to the different molecular chaperones were repeated, and the resultant immunoprecipitates were heated in Laemmli sample buffer containing extra reducing agent to reverse the cross-links before analysis by SDS-PAGE (Figure 5C). In each case, the different molecular chaperone antibodies were capable of capturing their respective antigens after the cross-linking reaction, and excessive cross-linking of cellular proteins to any one chaperone was not apparent. When the same cross-linking analysis was performed with pulse-labeled cells, antibodies to hsp70 again were the most efficient in coprecipitating the radiolabeled nascent chains (Figure 5D). However, now antibodies to the cytosolic chaperonin (CCT) and hsp90 also captured some nascent chains and/or newly synthesized proteins. In the case of CCT, one should note the coprecipitation of discrete bands representing proteins of approximately 45, 50–55, and 100 kDa in mass (Figure 5D, lane 2, +DSP). The two smaller proteins correspond in size to

full-length actin and tubulin (see next section). The nature of the 100-kDa band is unknown. When compared with control precipitations, little or no radioactive material was observed to coprecipitate with antibodies to the DnaJ homologue, hsp40, or to the 48-kDa protein (Figure 5C, lanes 3 and 4).

The coprecipitation of nascent polypeptides with hsp70, as shown in the two pulse-labeling experiments of Figure 5, B and D, should not be compared quantitatively. In the experiment of Figure 5B, puromycin was used to accumulate a pool of truncated nascent polypeptides throughout the 20-min labeling period. In contrast, the number of incomplete nascent polypeptides in the cross-linking experiment could not exceed the absolute number of active ribosomes (Figure 5D). Nevertheless, all three approaches shown in Figure 4 and Figure 5, B and D, implicate hsp70 as being the major class of molecular chaperones that interact with nascent polypeptides in the cytosol of HeLa cells. Hsp90 was found in complexes with nascent polypeptides only after stabilization of the interaction by cross-linking (Figure 5D, lane 5).

CCT Binds Newly Synthesized Full-Length Actin and Tubulin In Vivo

To identify the newly synthesized proteins that coprecipitated with CCT (Figure 5D, lane 2), the immunoprecipitation experiments were repeated, and the resultant immunoprecipitates were analyzed by two-dimensional gel electrophoresis. For these experiments, two plates of HeLa cells were labeled overnight and chased for 4.5 h. One plate was harvested immediately. Culture medium containing fresh [³⁵S]methionine was added to the other plate, and newly synthesized proteins were labeled for 15 min before lysing the cells. Immunoprecipitation reactions were performed under nondenaturing conditions using the antibody to the γ -subunit of CCT. From the steady-state labeled cells, only the subunits comprising the cytosolic chaperonin were precipitated (Figure 6B). Those subunits of CCT having more basic isoelectric points did not focus well in the pH range used for the first dimension. A trace amount of mature tubulin was observed to coprecipitate with CCT under these conditions. From those cells provided the second 15-min labeling period; however, discernible amounts of newly synthesized actin and (α , β)-tubulin were observed to coprecipitate with the cytosolic chaperonin (Figure 6C). As expected, more of the γ -subunit of CCT also was precipitated, consistent with the capture of newly synthesized antigen that had not yet assembled into mature CCT particles.

The binding of CCT to full-length actin and tubulin (Figure 6C), along with the lack of CCT found in a complex with puromycin-released polypeptides (Figure 4A), is consistent with the idea that the cytosolic

chaperonin does not bind to its substrates in a cotranslational manner. To address this issue directly, polysomes were isolated by velocity sedimentation and examined for the presence of cosedimenting chaperones. Analysis of the material by one-dimensional SDS-PAGE revealed numerous proteins, many of which corresponded in size to constituents of the large and small ribosomal subunits (Figure 6D, right). As expected, most of the basic ribosomal proteins were not resolved when analyzed by an equilibrium two-dimensional gel using ampholines in a slightly acidic pH range (Figure 6D, left). Proteins that fractionated with the polysomes included the cytosolic forms of hsp70, as well as a number of cytoskeletal proteins. The different protein constituents of CCT were not apparent within the isolated polysomes, suggesting that the cytosolic chaperonin does not interact with nascent polypeptides during their synthesis.

Puromycin-released Polypeptides Bind Nonspecifically to Mature Proteins after Cell Lysis

All of the results presented in this report indicate that members of the hsp70 family represent the most prominent molecular chaperones that interact with nascent polypeptides. However, significant amounts of both actin and tubulin consistently were observed to coprecipitate with the puromycin-released nascent chains. We suspected that the coprecipitation of these two abundant proteins may originate from nonspecific hydrophobic interactions with the nascent polypeptides because many of the truncated polypeptides are unable to fold into stable structures. To further characterize this interaction, the effect of reducing the overall protein concentration of the cell lysate before the immunoprecipitation analysis was examined for its consequences on nonspecific binding to puromycin-polypeptides. In the experiment shown in Figure 7, each sample was programmed with the same amount of steady-state labeled cell lysate, immunoprecipitated with the same amount of puromycin antibody, and washed under the same stringent conditions. The only difference between the individual reactions was the initial dilution of the cell lysate before addition of the antibody. In the sample, where the overall protein concentration was the highest (1:2 dilution), we observed the greatest amount of coprecipitating proteins (Figure 7, lane 2). Further lysate dilutions of 1:6, 1:20, and 1:60 resulted in a corresponding decrease in all coprecipitating proteins. At the highest dilution, hsp70 was clearly the major protein bound to puromycin-polypeptides (Figure 7, lane 5). The other coprecipitating proteins were, in fact, associated directly with the nascent polypeptides (and not binding nonspecifically to the antibody itself) because they were not precipitated when the antibody was blocked with free puromycin (compare Figure 7,

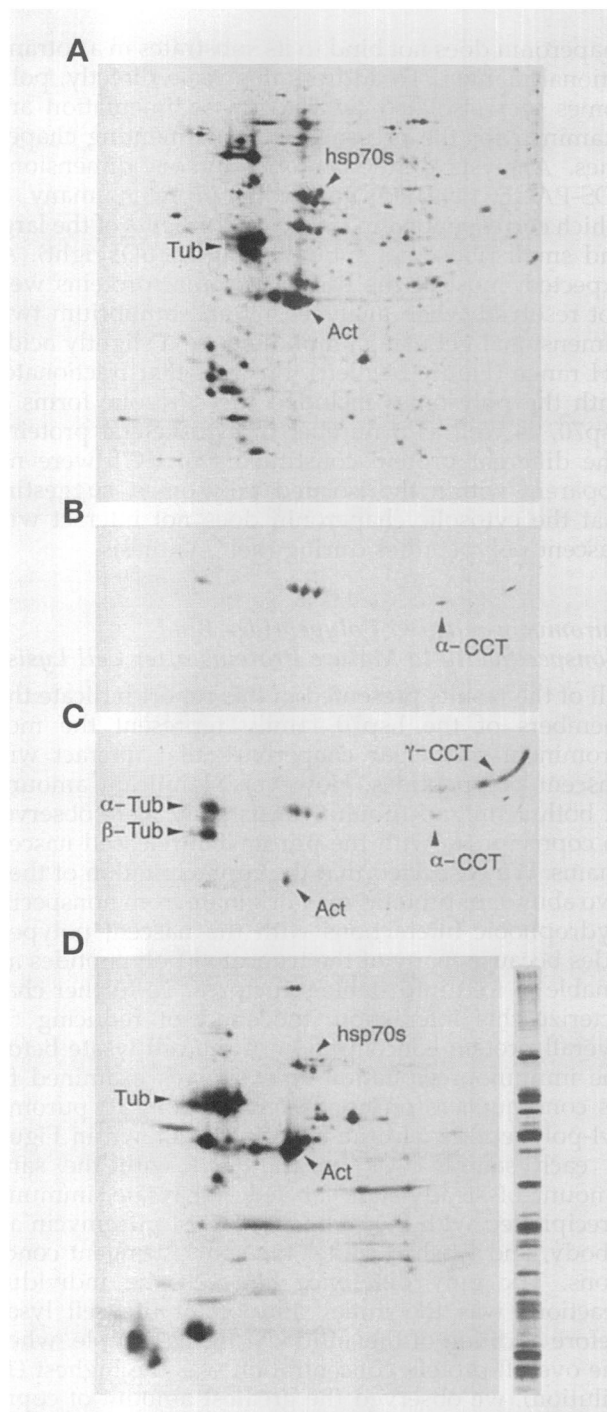


Figure 6. Complexes between CCT and newly synthesized full-length actin and tubulin after a 15-min pulse with [^{35}S]methionine. Two-dimensional gel analysis of (A) total protein from cells steady-state labeled with [^{35}S]methionine and lysed in detergent; (B) immunoprecipitation of CCT from a 1:20 dilution of a lysate prepared identically to that shown in (A); (C) immunoprecipitation of CCT from cells which were labeled overnight with [^{35}S]methionine, chased 4.5 h in the absence of label, and then labeled again with [^{35}S]methionine for 15 min just before lysis. (D) Two-dimensional profile of isolated polysomes from HeLa cells that were steady-state labeled with [^{35}S]methionine before hypotonic lysis (see Materials

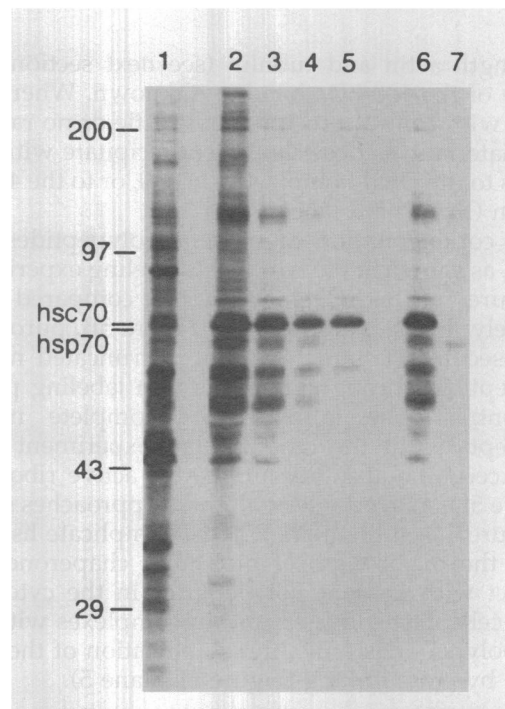


Figure 7. Nonspecific protein binding to puromycin-released nascent polypeptides as a function of protein concentration. HeLa cells were steady-state labeled with [^{35}S]methionine and treated with 2 μM puromycin for 15 min after the chase period. For each immunoprecipitation (lanes 2–7), 50 μl of the same lysate was used but diluted to different volumes with buffer A + 0.1% Triton X-100 before addition of the antipuromycin antibody. (1) Starting lysate; (2) immunoprecipitation after 1:2 dilution; (3) 1:6 dilution; (4) 1:20 dilution; (5) 1:60 dilution; (6) identical sample as lane 3, 1:6 dilution; (7) 1:6 dilution with addition of 100 μM puromycin to block antibody.

lanes 6 and 7). The increased detection of these proteins in more concentrated samples may be attributed to both an increase in nonspecific binding per nascent chain and an increase in the number of nascent chains captured under conditions of higher antibody/antigen concentrations (unpublished observations). It should be noted that the proteins identified in the two-dimensional gel of Figure 4A were coprecipitated from a lysate dilution of only 1:5, which explains the large amount of tubulin present in that particular sample.

Dynamics and Affinity of Hsp70 Interaction with Puromycyl-Polypeptides

With the exception of the cross-linking experiment, all of the previous results were dependent on the deple-

Figure 6 (cont). and Methods for details). A one-dimensional profile of the same polysome sample is shown at right. Note that most of the basic ribosomal proteins do not focus in the pH range used for the first dimension.

tion of ATP in the lysate before immunoprecipitation analysis to stabilize the interaction of hsp70 with the puromycin-polypeptides. To further examine the affinity of hsp70 for nascent polypeptides as a function of ATP levels, cells were disrupted in the presence of exogenous proteins, both native and abnormally folded. We rationalized that high concentrations of a nonnative protein, such as reduced and carboxymethylated BSA (RC-BSA) or α_s -casein, should compete with puromycin-polypeptides for binding of hsp70 under conditions that facilitate dynamic interactions. In those samples where ATP levels were depleted immediately with apyrase during cell lysis, significant amounts of hsp70 were observed to coprecipitate with the puromycin-released nascent chains independent of the presence of the different protein supplements (Figure 8A). A significant reduction in the "background" of other proteins that coprecipitated with the released nascent chains was observed, however, from the lysates containing RC-BSA, casein, or a sixfold excess of unlabeled HeLa cell lysate (Figure 8A, lanes 3–5, respectively).

Proteins that coprecipitated with the puromycin antibody from a control lysate and from a lysate prepared in 10 mg/ml of RC-BSA were compared by two-dimensional gel electrophoresis (Figure 8, D and E). Clearly, the presence of RC-BSA reduced the binding of tubulin and grp75 to puromycin-polypeptides without affecting the coprecipitation of hsc70 and hsp70. The reduction in background coprecipitation was not observed after immunoprecipitation from a sample where RC-BSA had been added subsequent to cell lysis and ATP depletion (unpublished observation). When present during cell disruption (i.e., in the lysis buffer), RC-BSA and casein seem to intercept many of the proteins that coprecipitated with nascent polypeptides in a concentration-dependent, nonspecific manner in Figure 7. These observations support the idea that hsp70 is the major relevant protein interacting with puromycin-polypeptides in the cytosol (before lysing the cells).

When ATP was present during cell lysis, labeled hsp70 no longer was observed to coprecipitate with the puromycin-released chains (Figure 8B). Instead, only those proteins shown to bind nonspecifically were coprecipitated. The amount of puromycin-released chains that were captured with the puromycin antibody was unaffected by ATP depletion or by the presence of any of the exogenous proteins, as quantified in a parallel experiment using pulse-labeled lysates (Eggers, 1997). In those lysates where ATP was initially present and then depleted after a 10-min incubation, rebinding of the radiolabeled hsp70 chaperone to the puromycin-released nascent chains was observed. The extent of rebinding, however, varied as a function of the particular protein supplement (Figure 8C). In those lysates supplemented with either nothing or native BSA, hsp70 was observed to coprecipitate again with the puromycin-released

chains (Figure 8C, lanes 1 and 2). In contrast, much less radiolabeled hsp70 was observed to coprecipitate with the puromycin-released chains in those lysates supplemented with 10 mg/ml of either RC-BSA or α_s -casein (Figure 8C, lanes 3 and 4). This is consistent with the idea that these nonnative protein supplements provide a vast excess of substrate for the hsp70 chaperone. Alternatively, after the ATP-dependent release of hsp70 from the puromycin chains, the interacting sites on the chains themselves may become bound to the excess of added RC-BSA or casein (but not by the native BSA), resulting in less rebinding of chaperones as well as minimizing nonspecific interactions with labeled proteins. In the case where unlabeled HeLa cell lysate was present (Figure 8C, lane 5), a significant reduction of the radiolabeled hsp70 chaperone was expected because the sixfold excess of unlabeled hsp70 could compete for rebinding to the puromycin-released nascent chains. Overall, the results of Figure 8 are consistent with the hypothesis that the interaction between members of the hsp70 family and nascent polypeptides is one of high affinity yet constant dynamics in the presence of physiological concentrations of ATP.

DISCUSSION

It has been established that nascent polypeptides interact with molecular chaperones during the early stages of their maturation. All of the available evidence indicates that molecular chaperones influence protein maturation by minimizing "off-pathway" folding events, including aggregation. The first chaperones shown to interact in a general way with nascent and newly synthesized proteins were members of the hsp70 family. The hsp70 chaperones, distributed throughout all intracellular compartments, seem to interact with nascent polypeptides still bound to the ribosome and with proteins as they are being transferred from the cytosol into the lumen of organelles. The interaction between a nascent polypeptide and its particular chaperone(s) is thought to stabilize transiently exposed hydrophobic regions on the polypeptide until enough amino acids are incorporated into the growing chain (or translocated through an organellar membrane) to allow for the folding of a stable structure.

Results of recent studies have led to the suggestion that the folding and maturation of newly synthesized proteins in the eukaryotic cytosol is facilitated by the sequential and cooperative interactions of molecular chaperones from multiple families. In the model of Hartl (1996), as a nascent polypeptide emerges from the ribosome, it is first recognized by and becomes bound to hsp40, a homologue of the bacterial DnaJ protein (Frydman *et al.*, 1994). DnaJ or hsp40 acts as a cofactor for the DnaK or cytosolic hsp70 chaperones, respectively, by stimulating their relatively weak ATPase activities. The

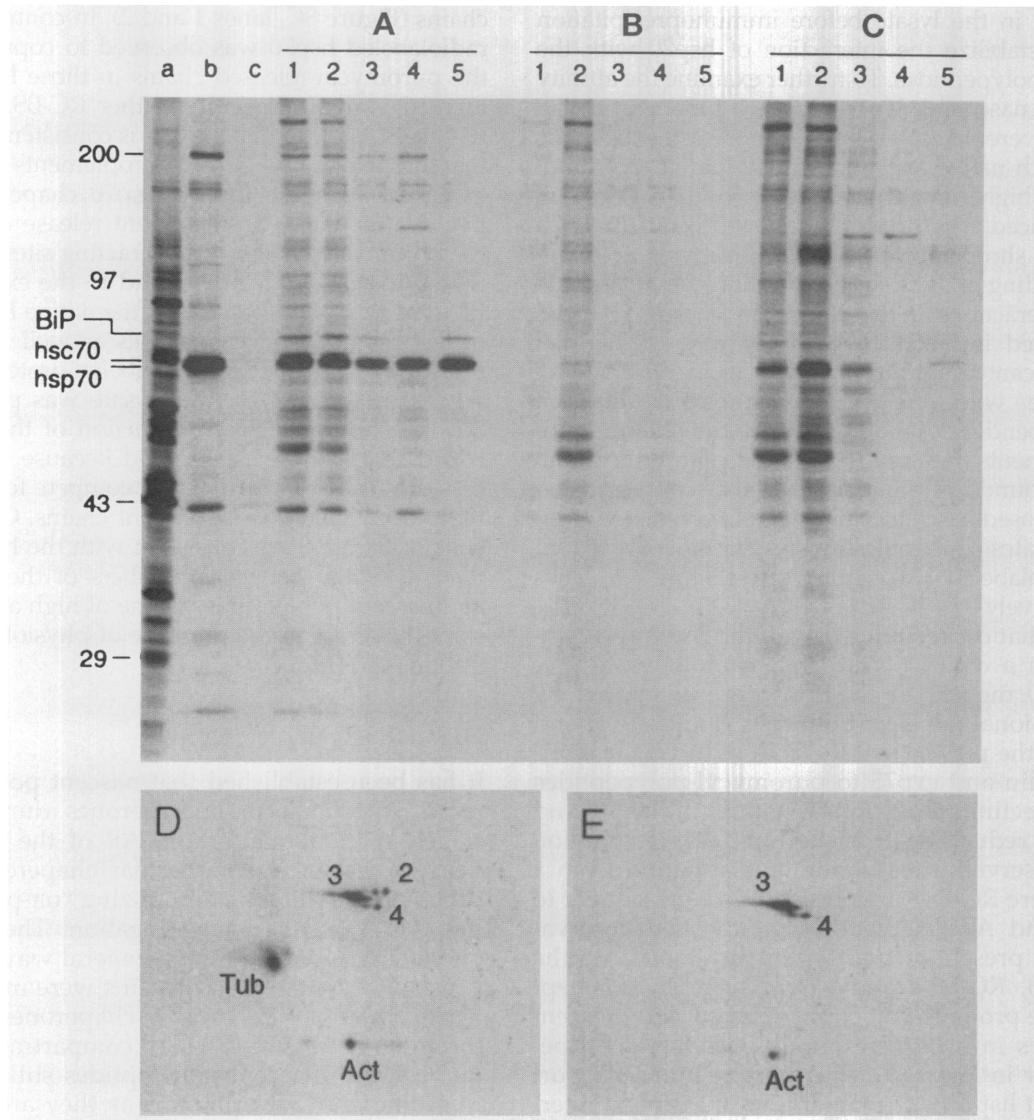


Figure 8. Affinity of hsp70 for puromycin-polypeptides in the presence of exogenous proteins. Cells were steady-state labeled with [35 S]methionine and treated with 2 μ M puromycin for 30 min. Cells were lysed in buffer A + 0.1% Triton X-100 supplemented with different exogenous proteins (numbered 1–5) and incubated with either (A) apyrase, (B) 10 mM MgATP, or (C) 10 mM MgATP for 10 min, followed by apyrase treatment. Control lanes on the left side of figure: (a) typical starting lysate; (b) immunoprecipitation with antibodies to hsc70/hsp70; (c) puromycin antibody blocked with free puromycin before immunoprecipitation. Antipuromycin immunoprecipitations were performed under nondenaturing conditions after 1:40 dilution of lysates made in the presence of the following supplements: (1) no exogenous protein; (2) 10 mg/ml BSA; (3) 10 mg/ml RC-BSA; (4) 10 mg/ml α_s -casein; (5) sixfold excess of unlabeled HeLa protein from cells untreated with puromycin. (D) Two-dimensional gel analysis of proteins that coprecipitated with puromycin-polypeptides from control cells lysed in the presence of apyrase and no exogenous protein. (E) Two-dimensional gel analysis of proteins that coprecipitated with puromycin-polypeptides from cells lysed in the presence of apyrase and 10 mg/ml RC-BSA. Protein identification numbers for the two-dimensional gels are the same as that in Figure 4: (2) grp75; (3) hsc70; (4) hsp70.

substrate affinity of hsp70 (i.e., the ratio of bound to unbound substrate at equilibrium) is reported to be higher when the chaperone is in the ADP-bound conformation, even though the kinetic rate constant for association with the substrate is higher for the ATP-bound conformation (Schmid *et al.*, 1994; McCarty *et al.*, 1995; Theyssen *et al.*, 1996). Thus, hsp40 would bind first to a

nascent polypeptide and presumably recruit hsp70 to the substrate with a bound molecule of ATP. Formation of the ternary complex would result in the activation of the ATPase activity of hsp70 (via the action of hsp40) and, therefore, in a more stable interaction between the hsp70 chaperone and the nascent polypeptide. Release of the hsp70 chaperone from the nascent chain substrate

would require the return of hsp70 to its ATP-bound state via a nucleotide exchange event. For some proteins, the nascent chain would be transferred from hsp70/hsp40 to another chaperone system, the high molecular weight cytosolic chaperonin complex (CCT), where synthesis and/or folding of the polypeptide would be completed.

We have presented results obtained from a combination of metabolic labeling and immunoprecipitation techniques, including the use of an antibody to puromycin, which were designed to verify the presence of various nascent polypeptide complexes that might be predicted from the preceding model. The use of puromycin differs from other reported methods for examining interactions with nascent polypeptides in two major ways: (1) puromycyl-polypeptides are synthesized as a population of randomly truncated polypeptides obtained from all the actively translated mRNAs in the cell, and, therefore, the complexes which are isolated and characterized should represent an average of all of the chaperone interactions that occur *in vivo*; and (2) the puromycin antibody allows one to capture these complexes without any experimental bias as to the identity of the bound chaperones before immunoprecipitation.

We were hopeful that the use of the puromycin antibody would lead to the discovery of new chaperones that had not been implicated previously in the process of protein folding and maturation. Instead, the well-known members of the hsp70 family were found to be the predominant species that coprecipitated with puromycyl-polypeptides (Figure 4). The higher degree of hsp70 binding relative to other selected chaperones was corroborated by the more traditional approach of using chaperone-specific antibodies to coprecipitate nascent polypeptides. Via pulse labeling and immunoprecipitation under conditions that maintain protein-protein interactions, antibodies specific for hsp70 were found to be the most effective for the coprecipitation of the puromycin-released nascent chains (Figure 5B). The cytosolic forms of hsp70 showed a strong affinity for puromycyl-polypeptides relative to RC-BSA or casein when cells were lysed in the presence of a huge excess of these two abnormally folded proteins and simultaneously depleted of ATP (Figure 8A).

One caveat of using puromycin is that some relevant chaperone interactions might be lost after release of the nascent polypeptide because they occur only in the context of a translating ribosome. For example, it has been reported that two nascent chain binding components, NAC and trigger factor, no longer can be detected in association with nascent chains that have been released prematurely from the ribosome via puromycin (Wiedmann *et al.*, 1994; Valent *et al.*, 1995; Hesterkamp *et al.*, 1996). Consequently, a cross-linking approach was used in the absence of puromycin treatment to address this issue. In those experiments using the membrane-permeable cross-linker DSP to stabilize

chaperone interactions before cell disruption, immunoprecipitation with antibodies to cytosolic hsp70 again resulted in the highest capture of nascent chains relative to other chaperones (Figure 5D). This result provides further evidence that hsp70 interacts with the nascent chains in a cotranslational manner and that this interaction may be captured without manipulating the pool of ATP (i.e., under conditions that promote dynamic binding and release of hsp70). It is of interest that, after chemical cross-linking, antibodies to hsp90 were effective in coprecipitating a population of the radiolabeled nascent polypeptides (Figure 5D, lane 5). Because hsp90 was not observed to bind to nascent chains by any other approach, we suspect that hsp90 interactions are sensitive to the method of cell lysis and immunoprecipitation in the absence of chemical cross-linking. Hsp90 has been shown previously to affect the refolding of model proteins *in vitro* (Jakob *et al.*, 1995; Freeman and Morimoto, 1996).

Throughout the course of these studies, we were unable to detect any obvious interaction of the DnaJ homologue, hsp40, with the nascent polypeptides. Antibodies to hsp40 failed to coprecipitate any nascent or newly synthesized proteins (with or without the use of chemical cross-linking). Similarly, hsp40 was not observed to coprecipitate when the puromycin-released nascent polypeptides were captured via the puromycin antibody. Because the isoelectric point of hsp40 is very basic (pI 8.9; Ohtsuka, 1993), the puromycin immunoprecipitates shown in Figure 4 also were analyzed by nonequilibrium two-dimensional gel electrophoresis, but no hsp40 was detected (unpublished observation). These results, coupled with other studies (Nagata *et al.*, manuscript in preparation), lead us to believe that hsp40 does not bind to nascent polypeptides. Furthermore, we did not observe any interactions between nascent polypeptides and the p48 (Hip) protein, another suggested chaperone and cofactor for hsp70 (Höhfeld *et al.*, 1995).

With respect to the cytosolic chaperonin, a significant amount of radioactive material was observed to coprecipitate with CCT after chemical cross-linking of cells that were labeled for only 5 min. For the most part, however, the coprecipitating material seemed to represent full-length proteins. Via analysis by two-dimensional gels, these full-length proteins were shown to include newly synthesized actin and tubulin (Figure 6). A similar interaction has been reported in CHO cells using a different antibody to CCT (Sternlicht *et al.*, 1993). Collectively, our data strongly support the view that CCT interacts posttranslationally with a restricted "target range" of binding partners *in vivo* (Lewis *et al.*, 1996).

When puromycin-treated HeLa lysates were analyzed by gel filtration and velocity sedimentation, many of the puromycin-released nascent chains exhibited native molecular masses much larger than their

apparent molecular mass as determined by SDS-PAGE (Figure 2, A and B). Even though nonspecific interactions may contribute to the large and heterogeneous size distribution, it should be noted that few of the puromycin-released nascent chains fractionated at a size that would indicate any interaction with CCT; native CCT is 800–950 kDa and sediments at 20 s (Frydman *et al.*, 1992; Lewis *et al.*, 1992). Results of limited proteolysis reactions revealed that many of the nascent proteins are maintained in a protected environment by virtue of their interaction with other cellular components (Figure 3). The fact that enhanced proteolysis of the nascent polypeptides was observed in those lysates supplemented with ATP is consistent with the known ATP dependence governing chaperone-substrate interactions, including those of the hsp70 family of chaperones.

It is important to point out that we routinely observed many of the puromycin-released chains interacting with other abundant cellular proteins, especially actin and tubulin (Figure 4A). These interactions were greatly reduced by appropriate dilution of the initial lysate before immunoprecipitation (Figure 7) or by lysing the cells in the presence of RC-BSA or casein (Figure 8). Consequently, we suspect that nascent polypeptides have a propensity to interact nonspecifically with other cellular components. Indeed, it is this type of potential problem that likely accounts for the necessity of molecular chaperones in the early stages of protein synthesis and folding. We believe that the hsp70 chaperone fulfills this requirement by shielding the nascent chain from the crowded environment of the cytoplasm. The interaction of hsp70 with the nascent chain, however, does not seem to be a static event. As a consequence of the relatively high levels of ATP in a healthy cell, the functional activity of hsp70 is likely characterized by repeated cycles of binding to and release from its nascent chain substrates.

Presumably, dynamic chaperone interactions would allow protein folding to occur in a cotranslational manner. For example, during the early stages of translation, the nascent chain may not yet contain a suitable number of amino acids to allow for its productive folding. At this point in its synthesis, the nascent chain would be shielded, and thereby stabilized, via its interaction with the hsp70 chaperone. As polypeptide synthesis proceeds, the hsp70 chaperone would continue to undergo ATP-dependent cycles of binding to and release from the growing polypeptide chain without impeding the folding process. Once a sufficient number of amino acids have been incorporated to allow for complete folding of the amino-terminal domain, folding would commence after the next round of hsp70 release. This stable domain structure would no longer represent a target for hsp70 interaction. For those proteins containing multiple protein domains, one could envision the process being repeated during

the course of synthesis and folding of each domain. For the majority of single domain proteins, however, we suspect that acquisition of the final folded state will occur only after the synthesis of the nearly completed polypeptide or after release of the full-length protein from the ribosome. By combining the *in vivo* approach described here with *in vitro* translation of specific model proteins, it should be possible to further elucidate the properties of nascent polypeptides and their interactions with molecular chaperones.

ACKNOWLEDGMENTS

D.K.E. acknowledges past support from the Genentech Foundation for Biomedical Sciences through the ARCS Foundation, Northern California Chapter. Antibodies to p48 were a gift from D.F. Smith (University of Nebraska Medical Center, Omaha, NE), and the P5 antibody to CCT was kindly donated by E.C. Joly and V. Bibor-Hardy (Institut du Cancer de Montreal, Montreal, Quebec, Canada). We thank A. Kabiling for expert help in completing the two-dimensional gels. This work was supported by grants to W.J.W. from the National Institutes of Health (5 R01 GM33551) and National Science Foundation (MCB-9421946).

REFERENCES

- Anfinsen, C.B. (1973). Principles that govern the folding of protein chains. *Science* 181, 223–230.
- Beck, S.C., and De Maio, A. (1994). Stabilization of protein synthesis in thermotolerant cells during heat shock. *J. Biol. Chem.* 269, 21803–21811.
- Becker, J., and Craig, E.A. (1994). Heat-shock proteins as molecular chaperones. *Eur. J. Biochem.* 219, 11–23.
- 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, 850–854.
- Blattler, D.P., Garner, F., Van Slyke, K., and Bradley, A. (1972). Quantitative electrophoresis in polyacrylamide gels of 2–40%. *J. Chromatogr.* 64, 147–155.
- Brown, C.R., Martin, R.L., Hansen, W.J., Beckmann, R.P., and Welch, W.J. (1993). The constitutive and stress inducible forms of hsp70 exhibit functional similarities and interact with one another in an ATP-dependent fashion. *J. Cell Biol.* 120, 1101–1112.
- Buchner, J. (1996). Supervising the fold: functional principles of molecular chaperones. *FASEB J.* 10, 10–19.
- Dobrzynski, J.K., Sternlicht, M.L., Farr, G.W., and Sternlicht, H. (1996). Newly synthesized β -tubulin demonstrates domain-specific interactions with the cytosolic chaperonin. *Biochemistry* 35, 15870–15882.
- Eggers, D.K. (1997). Characterization of nascent polypeptides and their molecular chaperones in mammalian cells. Ph.D. thesis, University of California, San Francisco.
- Freeman, B.C., and Morimoto, R.I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hsp110 have distinct roles in recognition of a non-native protein and protein refolding. *EMBO J.* 15, 2969–2979.
- Frydman, J., and Hartl, F.U. (1996). Principles of chaperone-assisted protein folding: Differences between *in vitro* and *in vivo* mechanisms. *Science* 272, 1497–1501.
- Frydman, J., Nimmesgern, E., Erdjument-Bromage, H., Wall, J.S., Tempst, P., and Hartl, F.U. (1992). Function in protein folding of

- TRiC, a cytosolic ring complex containing TCP-1 and structurally related subunits. *EMBO J.* 11, 4767–4778.
- Frydman, J., Nimmesgern, E., Ohtsuka, K., and Hartl, F.U. (1994). Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370, 111–117.
- Georgopoulos, C., and Welch, W.J. (1993). Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* 9, 601–634.
- Gething, M.J., and Sambrook, J. (1992). Protein folding in the cell. *Nature* 355, 33–45.
- Hansen, W.J., Lingappa, V.R., and Welch, W.J. (1994). Complex environment of nascent polypeptide chains. *J. Biol. Chem.* 269, 26610–26613.
- Hartl, F.U. (1996). Molecular chaperones in cellular protein folding. *Nature* 381, 571–580.
- Hendrick, J.P., and Hartl, F.U. (1995). The role of molecular chaperones in protein folding. *FASEB J.* 9, 1559–1569.
- Hendrick, J.P., Langer, T., Davis, T.A., Hartl, F.U., and Wiedmann, M. (1993). Control of folding and membrane translocation by binding of the chaperone DnaJ to nascent polypeptides. *Proc. Natl. Acad. Sci. USA* 90, 10216–10220.
- Hestkamp, T., Hauser, S., Lutcke, H., and Bukau, B. (1996). *E. coli* trigger factor is a prolyl isomerase that associates with nascent polypeptide chains. *Proc. Natl. Acad. Sci. USA* 93, 4437–4441.
- Höfheld, J., Minami, Y., and Hartl, F.U. (1995). Hip, a novel cochaperone involved in the eukaryotic hsc70/hsp40 reaction cycle. *Cell* 83, 589–598.
- Jakob, U., Lilie, H., Meyer, I., and Buchner, J. (1995). Transient interaction of hsp90 with early unfolding intermediates of citrate synthase. *J. Biol. Chem.* 270, 7288–7294.
- Joly, E.C., Tremblay, E., Tanguay, R.M., Wu, Y., and Bibor-Hardy, V. (1994). TRiC-P5, a novel TCP1-related protein, is localized in the cytoplasm and in the nuclear matrix. *J. Cell Science* 107, 2851–2859.
- Kubota, H., Hynes, G., and Willison, K. (1995). The chaperonin containing t-complex polypeptide 1 (TCP-1). *Eur. J. Biochem.* 230, 3–16.
- Kudlicki, W., Odom, O.W., Kramer, G., and Hardesty, B. (1994). Chaperone-dependent folding and activation of ribosome-bound nascent rhodanese. *J. Mol. Biol.* 244, 319–331.
- Lai, B.-T., Chin, N.W., Stanek, A.E., Keh, W., and Lanks, K.W. (1984). Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. *Mol. Cell. Biol.* 4, 2802–2810.
- Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M.K., and Hartl, F.U. (1992). Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* 356, 683–689.
- Lewis, S.A., Tian, G., Vainberg, I.E., and Cowan, N.J. (1996). Chaperonin-mediated folding of actin and tubulin. *J. Cell Biol.* 132, 1–4.
- Lewis, V.A., Hynes, G.M., Zheng, D., Saibil, H., and Willison, K. (1992). T-complex polypeptide-1 is a subunit of a heteromeric particle in the eukaryotic cytosol. *Nature* 358, 249–252.
- Lingappa, J.R., Martin, R.L., Wong, M.L., Ganem, D., Welch, W.J., and Lingappa, V.R. (1994). A eukaryotic cytosolic chaperonin is associated with a high molecular weight intermediate in the assembly of hepatitis B virus capsid, a multimeric particle. *J. Cell Biol.* 125, 99–111.
- McCarty, J.S., Buchberger, A., Reinstein, J., and Bukau, B. (1995). The role of ATP in the functional cycle of DnaK chaperone system. *J. Mol. Biol.* 249, 126–137.
- Milarski, K.L., Welch, W.J., and Morimoto, R.I. (1989). Cell cycle-dependent association of hsp70 with specific cellular proteins. *J. Cell Biol.* 108, 413–423.
- Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., and Craig, E.A. (1992). The translation machinery and 70 kD heat shock protein cooperate in protein synthesis. *Cell* 71, 97–105.
- Ohtsuka, K. (1993). Cloning of a cDNA for heat-shock protein hsp40, a human homologue of bacterial DnaJ. *Biochem. Biophys. Res. Commun.* 197, 235–240.
- Palleros, D.R., Welch, W.J., and Fink, A.L. (1991). Interaction of hsp70 with unfolded proteins: effects of temperature and nucleotides on the kinetics of binding. *Proc. Natl. Acad. Sci. USA* 88, 5719–5723.
- Prapapanich, V., Chen, S., Nair, S.C., Rimerman, R.A., and Smith, D.F. (1996). Molecular cloning of human p48, a transient component of progesterone receptor complexes and an hsp-70 binding protein. *Mol. Endocrinol.* 10, 420–431.
- Reid, B.G., and Flynn, G.C. (1996). GroEL binds to and unfolds rhodanese posttranslationally. *J. Biol. Chem.* 271, 7212–7217.
- Sadis, S., and Hightower, L.E. (1992). Unfolded proteins stimulate molecular chaperone hsc70 ATPase by accelerating ADP/ATP exchange. *Biochemistry* 31, 9406–9412.
- Safiejko-Mroccka, B., and Bell, P.B. (1996). Bifunctional protein cross-linking reagents improve labeling of cytoskeletal proteins for qualitative and quantitative fluorescence microscopy. *J. Histochem. Cytochem.* 44, 641–656.
- Schmid, D., Baici, A., Gehring, H., and Christen, P. (1994). Kinetics of molecular chaperone action. *Science* 263, 971–973.
- Schröder, H., Langer, T., Hartl, F.U., and Bukau, B. (1993). DnaK, DnaJ and GrpE form a cellular chaperone machinery capable of repairing heat-induced protein damage. *EMBO J.* 12, 4137–4144.
- Schumacher, R.J., Hurst, R., Sullivan, W.P., McMahon, N.J., Toft, D.O., and Matts, R.L. (1994). ATP-dependent chaperoning activity of reticulocyte lysate. *J. Biol. Chem.* 269, 9493–9499.
- Sternlicht, H., Farr, G.W., Sternlicht, M.L., Driscoll, J.K., Willison, K., and Yaffe, M.B. (1993). The t-complex polypeptide 1 complex is a chaperonin for tubulin and actin in vivo. *Proc. Natl. Acad. Sci. USA* 90, 9422–9426.
- Szabo, A., Langer, T., Schröder, H., Flanagan, J., Bukau, B., and Hartl, F.U. (1994). The ATP hydrolysis-dependent reaction cycle of the *E. coli* hsp70 system: DnaK, DnaJ, and GrpE. *Proc. Natl. Acad. Sci. USA* 91, 10345–10349.
- Theyssen, H., Schuster, H.P., Packschies, L., Bukau, B., and Reinstein, J. (1996). The second step of ATP binding to DnaK induces peptide release. *J. Mol. Biol.* 263, 657–670.
- Tian, G., Huang, Y., Rommelaere, H., Vandekerckhove, J., Ampe, C., and Cowan, N.J. (1996). Pathway leading to correctly folded β -tubulin. *Cell* 86, 287–296.
- Valent, Q.A., Kendall, D.A., High, S., Kusters, R., Oudega, B., and Lührink, J. (1995). Early events in preprotein recognition in *E. coli*: interaction of SRP and trigger factor with nascent polypeptides. *EMBO J.* 14, 5494–5505.
- Wiedmann, B., Sakai, H., Davis, T.A., and Wiedmann, M. (1994). A protein complex required for signal-sequence-specific sorting and translocation. *Nature* 370, 434–440.
- Willison, K., Lewis, V., Zuckerman, K.S., Cordell, J., Dean, C., Miller, K., Lyon, M.F., and Marsh, M. (1989). The t complex polypeptide 1 (TCP-1) is associated with the cytoplasmic aspect of Golgi membranes. *Cell* 57, 621–632.