Mammalian Cytosolic DnaJ Homologues Affect the hsp70 Chaperone−Substrate Reaction Cycle, but Do Not Interact Directly with Nascent or Newly Synthesized Proteins†

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ABSTRACT: Members of the hsp70 family of molecular chaperones interact with and stabilize nascent polypeptides during synthesis and/or translocation into organelles. The bacterial hsp70 homologue DnaK requires the DnaJ cofactor for its reaction cycle with polypeptide substrates. DnaJ stimulates the ATPase activity of the DnaK chaperone and thereby is thought to regulate the affinity of DnaK for its protein target. Herein we have analyzed some of the biochemical properties of two mammalian cytosolic DnaJ homologues, the hdj-1 and hdj-2 proteins. We were particularly interested in examining the proposal that DnaJ homologues are the first molecular chaperones to interact directly with nascent polypeptides. Nascent/newly synthesized proteins, nascent polypeptides released from the ribosome by puromycin, or polypeptides misfolded as a result of incorporation of an amino acid analogue were not found in complexes with either of the two HeLa cell DnaJ homologues. We still were unable to demonstrate any interactions between hdj-1p and nascent/newly synthesized proteins even after chemical cross-linking. We did find that hdj-1p, like bacterial DnaJ, stimulated the ATPase activity of hsp70. Stable complex formation between hsp70 and an unfolded polypeptide substrate in vitro was found to be reduced in the presence of hdj-1p and ATP. Thus, while hdj-1p likely does function as a cofactor for the hsp70 chaperone, having effects on hsp70's ATPase activity and conformation/oligomeric structure and the stability of hsp70−substrate complexes, it was not observed to interact directly with nascent/newly synthesized proteins. Rather, hdj-1p likely serves a regulatory role, governing the reaction cycle of hsp70 with polypeptide substrates.

Protein folding inside the cell is believed to involve the participation of accessory components which now are being referred to as molecular chaperones. While not conveying any direct information for the folding process per se, molecular chaperones appear to stabilize intermediates in the folding pathway and thereby reduce the possibility of protein misfolding and/or aggregation (for review, see refs *1*−*3*). Many of the members of the *h*eat *s*hock *p*rotein (hsp)[1] family have been shown to function as molecular chaperones. Normally present at modest or even high levels, hsp's accumulate to higher levels in cells subjected to different metabolic traumas, including heat shock treatment and other proteotoxic insults. In the normal cell, the constitutively expressed hsp's participate in the synthesis, folding, and higher ordered assembly of cellular proteins. Following heat shock and/or other proteotoxic events, the increased levels of the different heat shock proteins likely facilitate the repair or replacement of proteins damaged as a consequence of the particular metabolic insult.

The best characterized molecular chaperones are the hsp70 and hsp60 families of heat shock proteins. In both yeast and animals cells the hsp70 family comprises multiple members, distributed throughout all intracellular compartments. In bacteria there is only a single hsp70 chaperone, the DnaK protein. Members of the hsp70/DnaK family appear capable of discriminating between properly folded and unfolded polypeptides, binding preferentially to the latter (*4*). Thus, during the course of protein synthesis, or when proteins are being translocated into organelles, members of the hsp70 family are thought to bind to and thereby stabilize the unfolded nascent polypeptide. Once synthesis and/or translocation has been completed, the polypeptide commences folding presumably upon its release from the hsp70 chaperone. The hsp60 protein, present within mitochondria and chloroplasts, and represented by the GroEL protein in bacteria, also binds to unfolded polypeptides. This class of chaperones, now also being referred to as "chaperonins," are characterized by their distinct higher ordered structure,

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[1] Abbreviations: HS, heat shock; azc, L-azetidine 2-carboxylic acid; hdj-1p, human DnaJ homologue-1 protein; hdj-2p, human DnaJ homologue-2 protein; hsp, heat shock protein; BSA, bovine serum albumin; RCMLA, reduced and carboxymethylated lactalbumin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ydj-1p, yeast DnaJ homologue-1 protein; sec, secretory mutant; kar-2p, karyogamy mutant-2 protein (yeast homologue of BiP, the immunoglobulin heavy chain binding protein); APY, apyrase; SBTI, soybean trypsin inhibitor; ECL, enhanced chemiluminescence; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; 2-ME, 2-mercaptoethanol, ER, endoplasmic reticulum.

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consisting of one or two seven-membered ring-like structures, oftentimes found stacked one on top of the other. Unfolded polypeptides are believed to enter into the central cavity of the chaperonin. Here, in this restricted environment, the polypeptide substrate appears to undergo ATP-dependent conformational changes which eventually lead to its final folded state. Weakly related cytoplasmic homologues of hsp60, referred to as CCT, the *c*ytosolic *c*haperonin containing *T*CP-1, have been described (for review, see ref *5*), but so far they have been shown to be important for the folding/assembly of only a limited number of polypeptides (*6*−*10*).

Both of these two major classes of chaperones require cofactors for their normal activities (for review, see refs *1*−*3*). Germane to the present report are the different cofactors which appear to support the DnaK, or hsp70, chaperones (we will use hsp70 and DnaK interchangeably). For example, members of the hsp70 family comprise two domains: a highly conserved amino-terminal domain involved in the binding and hydrolysis of ATP, and a less well conserved carboxy-terminal domain believed to be responsible for the interaction of the chaperone with short, unstructured peptides or unfolded polypeptides (*11*, *12*). The reaction cycle of the hsp70 chaperone with its polypeptide substrates is thought to be controlled via the binding, hydrolysis, and release of adenine nucleotides (*4*, *13*−*15*). For example, hsp70 in its ATP-bound state exhibits a high "on and off" rate for small peptides (used as a tool to mimic an unfolded polypeptide substrate). Only in its ADP-bound state does the hsp70 chaperone appear to form a stable complex with the substrate. By itself, however, the hsp70 chaperone exhibits very weak ATPase activities. Consequently, a cofactor, referred to as the DnaJ protein, is believed to regulate the ATPase activities of the hsp70 chaperone. Thus, in one possible reaction cycle, DnaJ acts to stimulate the ATPase activity of the hsp70 chaperone when containing a bound molecule of ATP. Hydrolysis of ATP is thought to result in a conformational change such that the hsp70 chaperone, now with a bound molecule of ADP, forms a stable complex with the unfolded polypeptide substrate. Subsequent release of the hsp70 chaperone from the substrate appears to require a nucleotide exchange event, believed to be mediated by the bacterial GrpE protein. Replacement of ADP with ATP again would result in a conformational change within the hsp70 chaperone and the release of the polypeptide substrate. Now with a bound molecule of ATP, the hsp70 chaperone would be ready for another round of substrate interaction.

Eukaryotic homologues of the bacterial DnaJ protein are being identified at a rapid pace, and they are present in the cell wherever the different forms of hsp70 are located (*16*−*18*). To date, only a single eukaryotic homologue of the GrpE protein has been identified and shown to work exclusively with the mitochondrial (and presumably chloroplast) forms of hsp70 (e.g., grp75) (*19*, *20*). At least two eukaryotic DnaJ homologues are present within the cytosol and nucleus. Like their bacterial counterpart, the related cytosolic DnaJ homologues exhibit masses of approximately 40 kDa and are referred to as hdj-1p and hdj-2p (*h*uman *D*na*J*) (*21*−*24*). As was shown previously (*25*) and in the present study, hdj-1p is heat-inducible and therefore represents another member of the heat shock protein family (referred to as hsp40).

While there is general agreement that members of the hsp70 family are capable of recognizing and binding to nonnative polypeptides (or short peptides), there have also been suggestions that members of the DnaJ family also can recognize and bind to unfolded polypeptides. For example, when first denatured via exposure to a protein chaotrope, unfolded rhodanese and luciferase have been reported to interact with and form a complex with the bacterial DnaJ protein (*26*−*28*). In a study using in vitro translation, nascent luciferase chains (i.e., those still bound to the ribosome) were found to interact with the hdj-1p/hsp40 eukaryotic DnaJ homologue (*8*). Consequently, Hartl and colleagues have suggested that members of the DnaJ family are the first to interact with nascent and/or unfolded polypeptides, and that once bound they then act to recruit the particular hsp70 chaperone to the substrate (*3*, *8*, *27*). Herein we have examined whether this proposed pathway of chaperone interactions is generally operative in the early stages of protein synthesis in animal cells.

EXPERIMENTAL PROCEDURES

Materials. A monoclonal antibody (C92) specific for Hsp72 and a monoclonal antibody (N27) which recognizes both Hsp72 and Hsp73 have been described previously (*29*, *30*). A polyclonal antibody specific for Hsp73 has been described previously (*31*). A monoclonal antibody specific for hdj-2p was obtained from NeoMarkers (Fremont, CA). A rabbit antibody recognizing puromycin was previously characterized (*32*, *33*).

The hdj-1p (hdj-1 protein) used in biochemical studies was purified by using a published procedure (*34*). cDNA for hdj-1 (human homologue of DnaJ) (*21*, *22*) was cloned into the pET23 vector (*p*lasmid for *e*xpression by *T*7 RNA polymerase) and provided by W. Sullivan and D. Toft (Mayo Clinic, Rochester, MN). The hdj-1p (hdj-1 protein) was expressed in *Escherichia coli* (BL21(DE3)) using the T7 RNA polymerase promoter system (*33*) induced by IPTG (final, 1 mM). Bacterial pellets were rinsed with 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl and 1 mM EDTA, and stored at −70 °C. Inclusion bodies were collected from bacterial pellets by sonication and subsequent centrifugation and then purified by washing with 50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1% sodium deoxycholate. Pellets were resuspended in freshly made 6 M urea in 50 mM Tris/HCl, pH 7.4, containing 1 mM EDTA and then dialyzed against the same buffer overnight. After clarification, the protein solution was dialyzed against the refolding buffer (50 mM Tris/HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol) overnight. The refolded purified protein was used to prepare a polyclonal antibody in rabbits.

Cell Culture and Metabolic Labeling. HeLa cells were grown in DMEM supplemented with 10% calf serum in a humidified incubator (5% CO_2). For heat shock treatments, the cells at near confluence were incubated at 43 °C for 90 min. For steady-state labeling, the cells were washed three times with DMEM lacking methionine and then incubated with [^{35}S]methionine (NEN Translabel, 1000 Ci/mmol; 1 Ci = 37 GBq) in medium consisting of 97% methionine-free DMEM, 3% complete DMEM, and 5% calf serum for

approximately 14 h. For metabolic pulse−chase labeling experiments, the culture medium was removed, the cells were washed three times with DMEM lacking methionine, and the cells then were labeled and harvested as described in the figure captions. Where the use of the proline analogue L-azetidine 2-carboxylic acid (azc) is indicated, the cells were preincubated in DMEM medium containing 5 mM azc for 2 h, which was followed by radiolabeling with [^{35}S]methionine in methionine-free DMEM containing 5 mM azc.

Immunological Methods. Immunoprecipitation reactions were performed under either denaturing or nativelike conditions. For the former, cells were solubilized in Laemmli sample buffer containing 1% SDS, heated at 95 °C for 5 min. The chromatin was sheared via passage of the lysate through a 28-gauge needle, and the lysate was then clarified via centrifugation in an Eppendorf centrifuge. Aliquots of the cell lysate were diluted 10-fold with PBS containing 1% Triton X-100 and 1% sodium deoxycholate (RIPA(−)) such that the final SDS concentration was 0.1%. The diluted lysate was incubated with Sepharose 6B (to allow for nonspecific protein absorption) for 30 min at 4 °C, the beads were removed by clarification, and then the primary antibody of interest was added to the supernatant. Rabbit anti-mouse antibodies were used in those immunoprecipitation reactions utilizing mouse monoclonal antibodies. Capture of the final immune complexes was achieved via addition of protein A−Sepharose. Immune complexes bound to protein A−Sepharose were washed a minimum of 4 times with RIPA-(+) buffer (RIPA(−) supplemented with 0.1% SDS). Proteins present in the immune complexes were released via addition of Laemmli sample buffer and heating at 95 °C for 5 min.

Immunoprecipitation under nativelike conditions was used to examine protein−protein interactions. Cells were solubilized in PBS supplemented with 10 mM KCl and 2 mM $MgCl_2$ and containing 0.1% Triton X-100 (and either apyrase or ATP added as indicated in the figure caption). Following clarification, the lysates then were used for the immunoprecipitation analysis essentially as described above. Resultant immune complexes were washed four times with RIPA(−) and the proteins present in the immune complex were released via addition of Laemmli sample buffer. Transfer of proteins from SDS−PAGE and Western blotting were done as described previously (*35*).

One- and Two-Dimensional Gel Electrophoresis. One-dimensional SDS−PAGE was performed as described by Laemmli, but using gel formulations as previously described (*36*). Two-dimensional gel electrophoresis, using either equilibrium or nonequilibrium isoelectric focusing followed by SDS−PAGE, was done as described previously (*37*).

Chemical Cross-Linking Experiments: (A) In Vitro. HeLa cells growing in a 3.5-cm dish were incubated with 100 μM puromycin for 10 min at 37 °C. Cells then were washed three times with DMEM lacking methionine and further incubated at 37 °C for 4 min. [^{35}S]Methionine (125 μCi/0.5 mL) then was added to the culture medium. Cycloheximide was added 5 min later (final 200 μM), and the cells were immediately harvested by the addition of 20 mM 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, supplemented with 0.1% Triton X-100, 2 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 10 units/mL apyrase, and 200 μM cycloheximide. One-half of the lysate was incubated with the cross-linker 3,3′-dithiobissulfosuccinimidyl propionate (DTSSP) (1 mM, Pierce Chemicals, Rockford, IL) for 30 min at 24 °C. The other half of the lysate was mock incubated. Excess cross-linker was quenched by the addition of glycine, and the lysate was then adjusted to a final 1% SDS concentration. Following heating of the lysate at 80 °C, immunoprecipitation reactions under denaturing conditions were performed using antibodies to either Hsp73 or hdj-1p. The resultant immunoprecipitated proteins were resuspended in Laemmli sample buffer containing extra reducing agent (to reverse the cross-link), heated at 90 °C, and then analyzed on 5−20% gradient SDS−PAGE.

(B) In Vivo. HeLa cells growing in 3.5-cm dishes were labeled with 0.2 mCi [^{35}S]methionine in 1 mL of methionine-free DMEM for 5 min at 37 °C and then treated with 0.1 mg/mL 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 20−22 °C with the cell-permeable cross-linker dithio*bis*[succinimidyl propionate] (DSP, Pierce Chemicals) (2 mM) dissolved in PBS containing 0.1 mg/mL cycloheximide. The DSP solution was removed, and the cross-linking reaction was quenched by incubating the cells in 50 mM glycine/50 mM Tris, pH 7, for 10 min. The cells were lysed in Laemmli sample buffer without any reducing agent and heated for 10 min at 75−80 °C, and the lysates were used for denaturing immunoprecipitations.

ATPase Assay. ATPase activity of Hsp73 was determined from the amount of [α-^{32}P]ADP produced from [α-^{32}P]ATP at 30 °C. The reaction mixture contained 0.33 pmol of [α-^{32}P]ATP (3000 Ci/mmol; DuPont), 100 μM unlabeled ATP, 30 mM HEPES buffer (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.3 mg/mL BSA, and purified hdj-1p. Bovine Hsp73 (StressGen, Victoria, BC) then was added, and the reaction mixture was incubated for 15 min at 30 °C. EDTA (final 20 mM) was added to stop the reaction, and 2 μL samples from the reaction mixture were analyzed by thin-layer chromatography on polyethyleneimine (PEI)−cellulose thin-layer sheets (PEI−cellulose-F, EM Science, Cherry Hill, NJ). Chromatography was performed using 1 M formic acid/1 M LiCl (1:1, vol/vol) at room temperature (*38*) The resultant chromatogram was dried and analyzed by autoradiography. Quantitation of ADP formed was done by densitometry using NIH Image software.

Interactions of hdj-1p with Hsp73 and Denatured Protein. ^{125}I-Reduced and carboxymethylated lactalbumin (RCMLA) was prepared according to the manufacturer's instructions using Iodobeads (Pierce Chemicals) and Na^{125}I (17 Ci/mmol; DuPont). The radioiodinated protein was separated from the unincorporated ^{125}I by gel filtration through Sephadex G-25. Hsp73 (StressGen, Victoria, BC), purified hdj-1p (*34*), and ^{125}I-reduced and carboxymethylated lactalbumin (RCMLA) in the amounts indicated in the figure captions, were incubated in 20 mM HEPES (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, and 5 mM 2-ME. Reactions were carried out for 30 min at 30 °C in the absence or presence of nucleotides as indicated. In some cases, apyrase (final 20 U/mL) was added at the end of the reaction to hydrolyze any remaining ATP. In the experiment presented in Figure 7 the Hsp73 preparation was depleted of ATP/ADP by incubation at 20 °C for 15 min with immobilized apyrase. The Hsp73 was separated from the apyrase−Affigel beads by centrifugation.

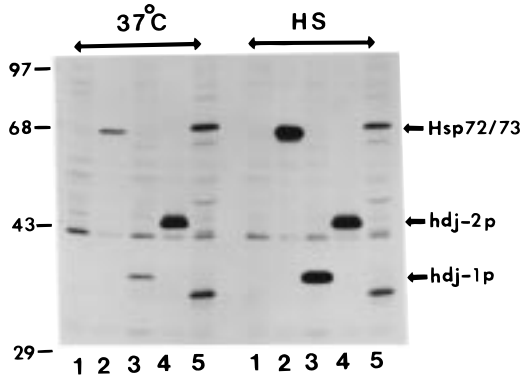

FIGURE 1: Specificity of the anti-molecular chaperone antibodies used in this study as determined by immunoprecipitation analysis. hdj-1, overexpressed in bacteria, was purified and used to raise a polyclonal antibody in rabbits as described in Experimental Procedures. To examine the specificity of the resultant sera, HeLa cells were either incubated at 37 °C or subjected to a 44 °C/30 min heat shock treatment. Following return of the heat shock treated cells to 37 °C, both sets of cells were incubated in culture medium containing [^{35}S]methionine. The cells were harvested 14 h later in Laemmli sample buffer, and the lysates were heated at 100 °C for 5 min. Immunoprecipitation reactions were performed, and the resultant immunoprecipitates were analyzed by SDS−PAGE. Lane 1, preimmune serum; lane 2, Hsp72 antibody; lane 3, hdj-1p antibody; lane 4, hdj-2p antibody; lane 5, Hsp73 antibody. Shown is a fluorograph of the gel. Lane designations are the same for both the 37 °C and the heat shock treated cells (HS). Molecular mass markers are indicated at the left.

Apyrase was immobilized by incubation with Affigel-10 (Bio-Rad, Richmond, CA) at 4 °C overnight, followed by quenching of the reactive sites on the beads with 50 mM ethanolamine, pH 7.5, and storage in PBS containing 50% glycerol at −20 °C. Analysis of the in vitro binding reactions was performed via native PAGE (*39*). Briefly, the native gel was composed of two parts: a 9% gel on the top and a 15% gel on the bottom. The upper chamber (cathode) buffer consisted of 50 mM Tricine and 15 mM BisTris, pH 7.0. The lower chamber (anode) buffer consisted of 50 mM BisTris, pH 7.0. Reaction mixtures were adjusted to a final 50 mM BisTris (pH 7.0) and 5% glycerol, and a small amount of tracking dye, Ponceau S, was added. The gel was run initially at 50 V for 20 min (approximately 5 mA) and then with a maximum current of 13 mA at 4 °C until the tracking dye migrated to the bottom of the gel. Proteins were visualized either by Coomassie Blue staining or by autoradiography.

RESULTS

hdj-1p, but not hdj-2p, Is a Heat Shock Protein with an Unusually Basic Isoelectric Point. Two mammalian cytosolic homologues of the bacterial DnaJ protein have been cloned and sequenced and are referred to as hdj-1p and hdj-2p (*21*−*24*). A rabbit polyclonal antibody was developed against recombinant hdj-1p overexpressed in and purified from bacteria. In addition, a monoclonal antibody to hdj-2p was obtained commercially. The specificity of both antibodies was examined via immunoprecipitation analysis using [^{35}S]methionine radiolabeled HeLa cell lysates (both normal and heat shock treated cells). The immunoprecipitation analysis was performed under "denaturing" conditions (cells lysed in a buffer containing SDS to disrupt all protein−protein interactions) to determine the specificity of the antibodies. As is shown in Figure 1, the rabbit polyclonal antibody raised against hdj-1p selectively immunoprecipitated an approximately 40-kDa protein. Following heat shock treatment, the levels of this 40-kDa protein increased significantly, consistent with previous reports that hdj-1p is a heat-inducible protein (*25*). The monoclonal antibody to the related hdj-2p antigen immunoprecipitated multiple protein species of approximately 45 kDa. Following heat shock treatment, the relative levels of hdj-2p did not increase. Using quantitative Western blotting, with purified Hsp73 and recombinant hdj-1p as standards, we estimate that the level of hdj-1p in HeLa cells growing at 37 °C is approximately 16-fold less than that of Hsp73 and Hsp72. After heat shock treatment, the ratio changes somewhat, with hdj-1p levels now being about $^{1}/_{10}$ that of the Hsp73/72 chaperones.

hdj-1p has been reported to exhibit a very basic isoelectric point (*22, 25*). To confirm that the antigen being recognized by our polyclonal antibody was indeed hdj-1p, we analyzed the cell lysates and the hdj-1p immunoprecipitates shown in Figure 1 by two-dimensional gel electrophoresis. Such an analysis revealed hdj-1p to exhibit an isoelectric point greater than 9.0 (data not shown). Thus, our antibody appears to be specific for hdj-1p, a protein that exhibits an unusually basic isoelectric point and whose expression increases in cells subjected to heat shock treatment (and which therefore is often referred to as hsp40). Although the data are not shown, both hdj-1p and hdj-2p were found to localize predominantly within the cytoplasmic fraction of mammalian cells.

Examining the Interaction of hdj-1p and hdj-2p with Nascent and Newly Synthesized Proteins. To determine whether hdj-1p or hdj-2p interacted with other polypeptides, immunoprecipitation experiments were performed. This time, however, the cells were harvested under conditions chosen to minimize the destruction of relevant protein−protein interactions. Both HeLa cells and monkey CV-1 cells, growing at 37 °C or following a heat shock treatment, were labeled to steady state with [^{35}S]methionine. The cells were harvested in phosphate-buffered saline supplemented with 0.1% non-ionic detergent, and immunoprecipitation experiments were carried out using antibodies to hdj-1p and hdj-2p. Antibodies specific for both Hsp73 and Hsp72 also were included in the analysis (Figure 2). From the HeLa cells maintained at 37 °C, low levels of hdj-1p were captured. Now under these milder conditions of cell lysis, two other proteins of approximately 60 and 65 kDa also were observed within the hdj-1p immunoprecipitates (Figure 2A, 37, lane 3). At the present time the identity of these two proteins is unknown. Presumably they represent proteins which either interact with hdj-1p or are immunologically related to hdj-1p (e.g., other DnaJ homologues), or both. The monoclonal antibody to hdj-2p was efficient in the capture of its appropriate antigen (Figure 2A, 37, lane 4). Other than the hdj-2p antigen itself, no other proteins were observed to coprecipitate. Note however, that the hdj-2p immunoprecipitation product migrated in the gel as an apparent doublet. Others have reported that the sequence of hdj-2p contains a potential prenylation site (*23, 24, 40*). Consequently, we suspect that a portion of the protein is prenylated and that this is what accounts for the observed doublet on the gel. Similar results were obtained for the monkey CV-1 cells maintained at 37 °C (Figure 2B). Briefly, hdj-1p, along with the 60- and 65-kDa antigens, were precipitated using the hdj-1p polyclonal antibody (Figure 2B, 37, lane 3). In the case

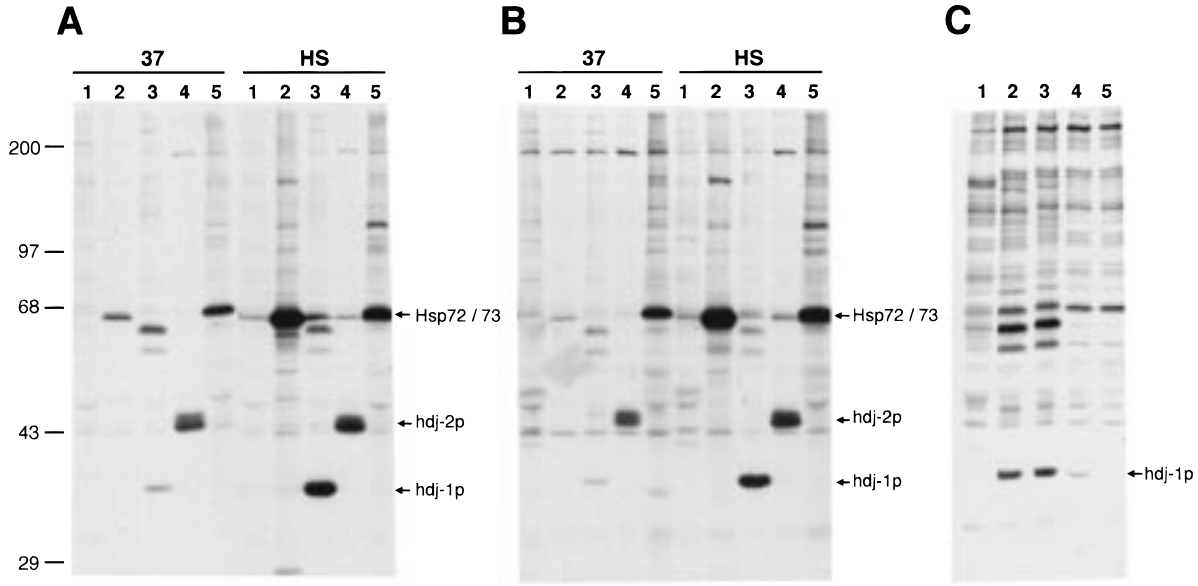

FIGURE 2: Cytosolic hdj proteins do not exhibit any significant interaction with hsp70 or other proteins as determined by immunoprecipitation. HeLa cells (A) and CV-1 cells (B) were maintained at 37 °C or given a 43 °C/90 min heat shock treatment. The cells were returned to 37 °C, and both sets of cells then were labeled with [^{35}S]methionine for 14 h at 37 °C. The cells were harvested in PBS supplemented with 10 mM KCl, 2 mM $MgCl_2$, and 0.1% Triton X-100. Apyrase was added to the lysate to deplete endogenous ATP levels. Immunoprecipitation reactions employing antibodies specific for hdj-1p, hdj-2p, Hsp72, or Hsp73 were performed from the normal (37) and heat shock treated (HS) cell lysates. The resultant immunoprecipitates were analyzed on 10% SDS−PAGE. Shown is a fluorograph of the gel. Lane designations are the same in each panel: lane 1, preimmune serum; lane 2, Hsp72 antibody; lane 3, hdj-1p antibody; lane 4, hdj-2p antibody; lane 5, Hsp73 antibody. Molecular mass markers are indicated at the left. (C) Preabsorption of hdj-1p antibody with purified antigen blocks the immunoprecipitation of hdj-1p, as well as the 60- and 65-kDa antigens. Rabbit anti-hdj-1p was diluted in a buffer containing either purified hdj-1p or bovine serum albumin (BSA) as described in Experimental Procedures. The preabsorbed antibody then was used in immunoprecipitation reactions using the lysate from HeLa cells labeled with [^{35}S]methionine at 37 °C (as described above). Resultant immunoprecipitates were analyzed on 10% SDS−PAGE. Shown is a fluorograph of the gel. Lane 1, preimmune serum; lane 2, hdj-1p antibody preabsorbed with BSA (diluted 1:1.5); lane 3, hdj-1p antibody preabsorbed with BSA (diluted 1:3.5); lane 4, hdj-1p antibody preabsorbed with purified hdj-1p (diluted 1:1.5); lane 5, hdj-1p antibody preabsorbed with purified hdj-1p (diluted 1:3.5).

of hdj-2p an apparent doublet again was observed (Figure 2B, 37, lane 4). Note that no other proteins, including Hsp73 and Hsp72, were observed to coprecipitate with either of the two DnaJ homologues. Similarly, neither hdj-1p nor hdj-2p was observed to coprecipitate when the analysis was performed using antibodies to either Hsp72 or Hsp73 (Figure 2A, B, 37, lanes 2 and 5, respectively).

Heat shock treatment had little or no effect on the pattern of proteins being precipitated by either the hdj-1p or hdj-2p antibodies. While the levels of hdj-1p increased significantly, no new polypeptides were found to coprecipitate with hdj-1p as a function of the heat shock treatment (Figure 2A, B, HS, lane 3). While the 60- and 65-kDa antigens were still observed, their levels did not appear to increase following the heat shock treatment. Heat shock treatment also had little or no effect on the hdj-2p immunoprecipitates (Figure 2A, HS, lane 4).

To determine whether the 60- and 65-kDa antigens being recognized by the hdj-1p antiserum might be due to a contaminating antibody present within our rabbit polyclonal serum, a blocking experiment using purified hdj-1p was performed. The hdj-1p sera were incubated with two different amounts of purified hdj-1p. As a control, the sera also were incubated with equivalent amounts of bovine serum albumin (BSA). Immunoprecipitation experiments using the sera preabsorbed with BSA had little or no effect on the pattern of precipitated proteins (Figure 2C, lanes 2 and 3). Again, from the steady-state radiolabeled HeLa cells, hdj-1p, as well as the 60- and 65-kDa antigens, was observed to be specifically captured. In contrast, incubation of the hdj-1p sera with the purified hdj-1p antigen now resulted in a failure of the serum to immunoprecipitate hdj-1p (Figure 2C, lanes 4 and 5). Note that now the 60- and 65-kDa antigens no longer were precipitated by the preabsorbed serum (Figure 2C, lanes 4 and 5). Thus, we suspect the immunoisolation of the 60- and 65-kDa antigens by the hdj-1p antiserum is not due to the presence of a contaminating antibody. Rather we believe that the 60- and 65-kDa antigens are related to hdj-1p and/or they interact specifically with the hdj-1p antigen in the cell. We currently are trying to obtain partial amino acid sequence information for these two unidentified polypeptides.

Frydman et al. have reported that hdj-1p is the first component to interact with a nascent polypeptide emerging from the eukaryotic ribosome (*8*). Once bound, hdj-1p then acts to recruit the cytosolic hsp70 chaperone to the growing polypeptide chain. To test whether the DnaJ homologues might interact with nascent or newly synthesized proteins in vivo, HeLa cells were subjected to pulse−chase radiolabeling, and antibodies to either hdj-1p, hdj-2p, or Hsp73/72 were compared for their ability to coprecipitate the radiolabeled nascent polypeptides. As we have shown previously (*41*), a portion of the newly synthesized, radiolabeled proteins were observed to coprecipitate with the Hsp73/72 chaperones in those cells pulse-labeled with [^{35}S]methionine for 20 min (Figure 3A, Hsp73, lane 1). When the cells were allowed a subsequent chase period in the absence of the radiolabel, the overall levels of radiolabeled polypeptides coprecipitating with the Hsp73 chaperone declined somewhat (Figure 3A, Hsp73, lanes 2 and 3). Note, however, that due to the

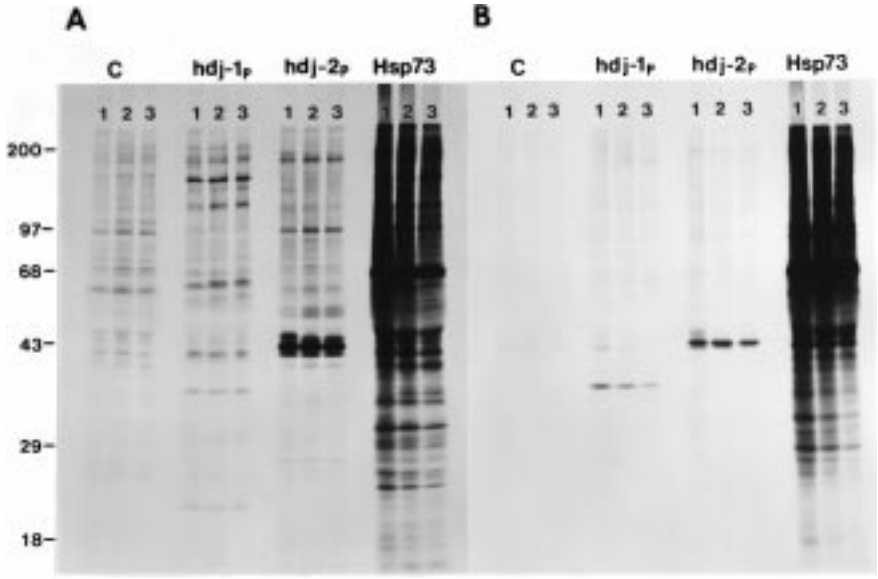

FIGURE 3: Unlike Hsp73/72, hdj-1p and hdj-2p do not interact with newly synthesized proteins. HeLa cells were incubated at 37 °C either in normal growth medium or in medium supplemented with 5 mM proline analogue L-azetidine 2-carboxylic acid (azc). The cells then were pulse-labeled for 20 min with [^{35}S]methionine. One plate of the labeled cells was harvested immediately. The medium was removed from the other radiolabeled cells, and the cells were washed with and incubated further in fresh culture medium not containing any radiolabel for either 30 or 60 min (i.e., pulse-labeled and chased). In each case, cycloheximide (40 μg/mL) was added during the chase period. The cells were lysed and used for immunoprecipitation analysis as described in Experimental Procedures. The resultant immunoprecipitates were analyzed by SDS−PAGE. Shown are fluorographs of the gels. Panel A shows the results from the cells maintained at 37 C, while panel B shows the results from the cells incubated in the presence of the amino acid analogue, azc. Lane designations are the same in each panel: preimmune serum (C), hdj-1 serum (hdj-1p), hdj-2 serum (hdj-2p), and Hsp73 (Hsp73). Lane 1, cells pulse-labeled for 20 min; lane 2, cells pulse-labeled for 20 min and then chased for 30 min; lane 3, cells pulse-labeled and chased for 60 min. Molecular mass markers are indicated at the left.

presence of the translational elongation inhibitor (cycloheximide) during the chase period some of the radiolabeled nascent polypeptides were still bound to their chaperone, even after the 30- or 60-min chase periods. Antibodies to either of the DnaJ homologues, hdj-1p or hdj-2p, were ineffective for the coprecipitation of nascent/newly synthesized proteins (Figure 3A, hdj-1p or hdj-2p). Under these conditions of immunoprecipitation only the relevant antigen (i.e., hdj-1p or hdj-2p), along with the same background of proteins observed using preimmune serum (Figure 3A, C), was present in the immunoprecipitates.

Our previous work has shown that proteins synthesized in the presence of an amino acid analogue exhibit relatively long lived interactions with their Hsp73 chaperone (*41*, *42*). We suspect that the incorporation of the amino acid analogue into a nascent polypeptide interferes with its proper folding. As a consequence, the abnormally folded protein probably remains as a substrate for the Hsp73 chaperone, likely until its eventual degradation (*43*). To determine whether the incorporation of an amino acid analogue into the nascent polypeptides might influence their possible interaction with DnaJ homologues, a pulse−chase experiment was performed in the presence of the amino acid analogue of proline, L-azetidine 2-carboxylic acid (azc). As is shown in Figure 3B, a significant number of the proteins synthesized in the presence of the amino acid analogue were found in a complex with the Hsp73 chaperone. None of the radiolabeled proteins synthesized in the presence of the amino acid analogue, however, were found to coprecipitate with either hdj-1p or hdj-2p (Figure 3B, hdj-1p or hdj-2p). [Note that the experiments shown in panels A and B of Figure 3 were done at different times. Hence, the different exposures of the films being presented account for the difference in the relative intensity of the background proteins being coprecipitated (for each panel, compare the background proteins in the hdj-1p, hdj-2p, and Hsp73 immunoprecipitates with the control immunoprecipitations utilizing preimmune serum within the same panel).]

As another approach to detect or trap hdj-1p nascent chain complexes, we examined whether hdj-1p might interact with nascent polypeptide chains released prematurely from the ribosome via the actions of the antibiotic puromycin. In the first experiment, HeLa cells were labeled to steady state with [^{35}S]methionine (to radiolabel a pool of molecular chaperones). The medium containing the radiolabel was removed, and the cells were incubated further in fresh culture medium to allow for the maturation of all of the radiolabeled proteins. Puromycin then was added to the cells to elicit the release of the nascent (not radiolabeled) polypeptides. After 20 min, the cells were harvested in a buffer containing non-ionic detergent and the cell lysate was split in half. ATP levels were depleted from one half of the lysate via incubation with the enzyme apyrase, while ATP was added to the other half of the lysate. After a 20-min incubation on ice, the puromycin-released nascent chains then were isolated using an antibody we prepared against puromycin (*32*, *33*). Radiolabeled proteins coprecipitating with the (unlabeled) puromycin-released nascent polypeptides were analyzed by SDS−PAGE (Figure 4A). In the those cell lysates where ATP levels had been depleted, the Hsp73/72 chaperones, along with a few other proteins currently under study in our lab, were found to coprecipitate with the puromycin-released nascent chains (Figure 4A, lane 2). In those lysates where ATP had been added, however, the relative amounts of Hsp73/72 which coprecipitated with the puromycin-released nascent polypeptides were markedly reduced (Figure 4A, lane

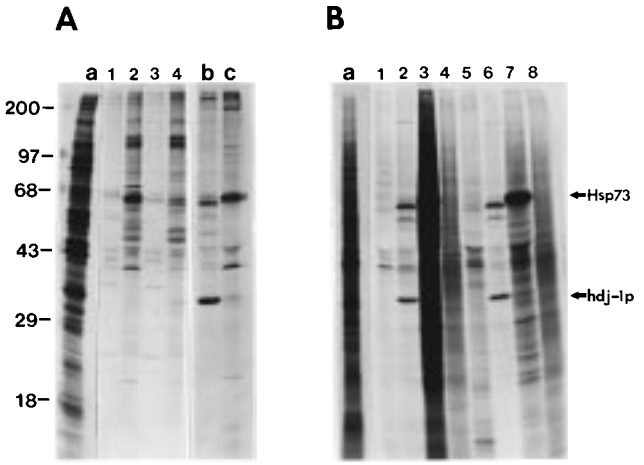

FIGURE 4: Hsp73, but not hdj-1p, interacts with puromycin-released nascent polypeptides. (A) HeLa cells growing at 37 °C were labeled with [^{35}S]methionine for 14 h. The culture medium containing the radiolabel was were removed, and the cells washed with and further incubated in fresh culture medium without radiolabel for 5 h at 37 °C. Puromycin then was added to the cells (final 2 μM), and after 20 min the cells were harvested in buffer supplemented with 0.1% Triton X-100. The cell lysate was divided in half: apyrase was added to one half of the lysate to deplete endogenous ATP levels; ATP was added to the other half of the lysate (final 10 mM). The cell lysates then were used for immunoprecipitation reactions using either rabbit preimmune serum or rabbit anti-puromycin (lanes 1−4). Immunoprecipitation reactions (from steady-state labeled cells) using antibodies to Hsp73 and hdj-1p are included to define the positions of both antigens. Lane a, HeLa cell lysate; lane b, hdj-1; lane c, Hsp73 immunoprecipitates included for reference; lane 1, immunoprecipitate using preimmune serum (ATP-depleted lysate); lane 2, anti-puromycin immunoprecipitate (ATP-depleted lysate); lane 3, immunoprecipitate using preimmune serum (ATP-supplemented lysate); lane 4, anti-puromycin antibody (ATP-supplemented lysate). The resultant immunoprecipitates were analyzed by SDS−PAGE. Shown are fluorographs of the gels. Molecular mass markers are indicated at the left, and to the right are arrows indicating the positions of Hsp73 and hdj-1p. (B) HeLa cells growing at 37 °C were incubated with 2 μM puromycin and [^{35}S]methionine for approximately 30 min as described in Experimental Procedures. The cells were harvested as described above and analyzed via immunoprecipitation using preimmune serum and antibodies specific to hdj-1p, Hsp73, or puromycin. Lane a, HeLa cell lysate used for the immunoprecipitation analysis; lane 1, preimmune serum (ATP-depleted lysate); lane 2, hdj-1p antibody (ATP-depleted lysate); lane 3, Hsp73 antibody (ATP-depleted lysate); lane 4, puromycin antibody (ATP-depleted lysate); lane 5, preimmune serum (ATP-supplemented lysate); lane 6, hdj-1p antibody (ATP-supplemented lysate); lane 7, Hsp73 antibody (ATP-supplemented lysate); lane 8, puromycin antibody (ATP-supplemented lysate). Note that in lanes 4 and 8 (anti-puromycin antibody), the volume of the radiolabeled cell lysate used for the immunoprecipitation analysis was approximately 1/20 that used for the other reactions.

4). In neither case (plus or minus ATP) were we able to detect hdj-1p coprecipitating with the puromycin-released nascent polypeptides.

In a parallel experiment, the abilities of the Hsp73 and hdj-1p antibodies to immunoprecipitate radiolabeled, puromycin-released nascent chains were compared. For this experiment HeLa cells were first incubated with 2 μM puromycin. Ten minutes later, [^{35}S]methionine was added to the culture medium and the cells were incubated further for an additional 20 min to generate a collection of radiolabeled, puromycin-containing nascent polypeptides. The cells were harvested, and the lysate was split in half; apyrase was added to one half of the lysate, while ATP was added to the other half. Immunoprecipitation experiments using antibodies to Hsp73 or hdj-1p were performed, and the resultant immunoprecipitates were analyzed by SDS−PAGE (Figure 4B). In those lysates first depleted of ATP, a significant amount of the puromycin-released, radiolabeled nascent polypeptides was observed to coprecipitate with Hsp73 (Figure 4B, lane 3). In contrast, addition of ATP to the cell lysate prior to the immunoprecipitation resulted in a marked decrease in the overall amount of radiolabeled polypeptides coprecipitating with the Hsp73 chaperone (Figure 4B, lane 7). Regardless of ATP levels, we were unable to detect any of the radiolabeled, puromycin-released nascent chains to coprecipitate with hdj-1p (Figure 4B, lanes 2 and 6).

Because the putative interaction of DnaJ homologues with nascent/newly synthesized proteins might be very transient and/or relatively labile, we turned to chemical cross-linking methods to stabilize the nascent chains in a complex with their relevant molecular chaperone(s). In the first approach radiolabeled, puromycin-released nascent chains were generated in HeLa cells as was described above. The cells then were lysed in a buffer containing low levels of non-ionic detergent, and the lysate was divided in half. The thiol-reversible cross-linker DTSSP was added to one half of the lysate, and the cross-linking reaction was allowed to proceed for 30 min before being quenched. The other half of the lysate was not exposed to the cross-linker, thereby serving as a control. Concentrated Laemmli sample buffer lacking any reducing agent was added to the lysates, and the lysates were heated at 75 °C for 5 min and then used for immunoprecipitation analysis. After dilution of the lysates (to reduce the SDS concentration), immunoprecipitation reactions were carried out using antibodies to either Hsp73 or hdj-1p. Proteins present in the immunoprecipitates were released by the addition of Laemmli sample buffer (lacking any reducing agent to maintain the cross-link) and analyzed by SDS−PAGE (Figure 5A). Examination of the cell lysates by SDS−PAGE revealed that the cross-linking reaction was effective. For example, the puromycin-released polypeptides from the control reaction (i.e., no added cross-linker) migrated in the gel as a smear of radioactive material with apparent masses of 70 kDa or less (Figure 5A, lane b). After cross-linking, however, the radiolabeled, puromycin-released polypeptides were observed to migrate from the very top of the gel down to the bottom (Figure 5A, lane a). Immunoprecipitation using antibodies to Hsp73 revealed that a significant amount of the puromycin-released polypeptides had been cross-linked to Hsp73 (Figure 5A, lane 2). Little or none of the radiolabeled, puromycin-released nascent chains, however, were observed to coprecipitate with hdj-1p (Figure 5A, lane 3).

In the next experiment, HeLa cells were incubated with [^{35}S]methionine for 5 min, and cycloheximide, an inhibitor of polypeptide chain elongation, was added to stabilize those nascent chains still bound to the ribosome. A cell-permeable and thiol-reversible chemical cross-linker, DSP, was added; the cross-linking reaction was allowed to proceed; and then the cells were prepared for immunoprecipitation analysis as described above. Resultant immunoprecipitates were solubilized in Laemmli sample buffer containing an excess of reducing agent to reverse the cross-link, and the precipitated proteins were analyzed via SDS−PAGE. Again, only

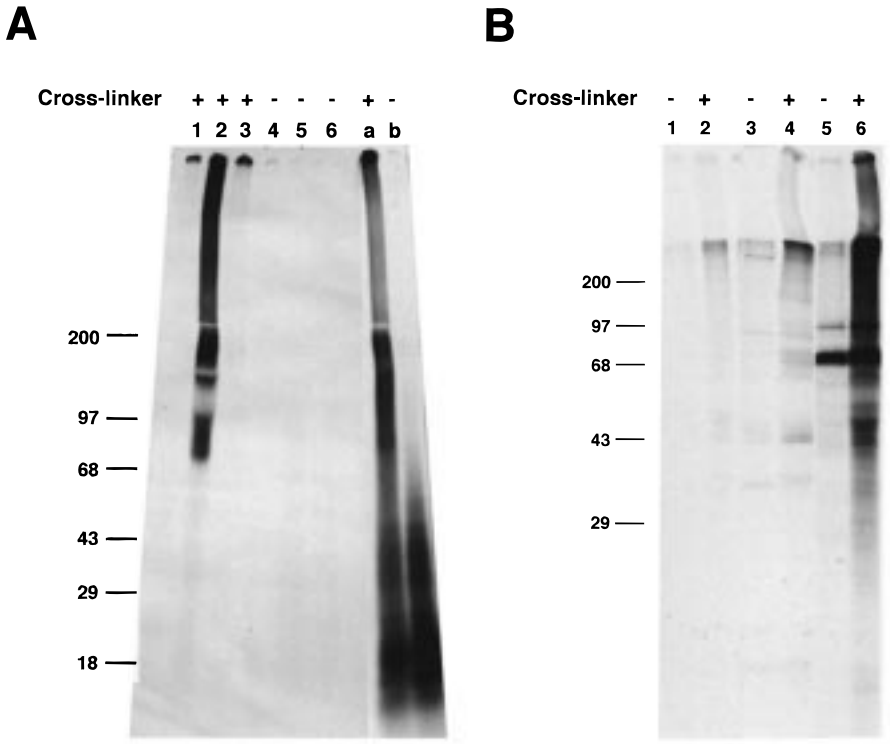

FIGURE 5: Analysis of nascent chain−chaperone interactions via chemical cross-linking. (A) HeLa cells were treated with 100 μM puromycin, the medium was replaced by medium lacking puromycin for 4 min, and the cells were labeled with [^{35}S]methionine for 5 min as described in Experimental Procedures. One half of the lysate was incubated with the cross-linker DTSSP as described in Experimental Procedures, with the other half serving as the control. Cell lysate proteins treated with or without DTSSP were denatured and used for immunoprecipitation by antibodies specific to hdj-1p or Hsp73. The immunoprecipitated proteins solubilized in nonreducing Laemmli sample buffer were analyzed on 5−20% gradient SDS−PAGE. Shown is a fluorograph of the gel. Lanes 1 and 4, preimmune serum; lanes 2 and 5, Hsp73 antibody; lanes 3 and 6, hdj-1p antibody. The cell lysate used for the immunoprecipitations shown in lanes 1−3 was incubated with 1 mM DTSSP, and this starting material is shown in lane a. The control lysate starting material (−DTSSP) shown in lane b was used for the immunoprecipitations shown in lanes 4−6. Molecular mass markers are indicated to the left. (B) HeLa cells were incubated with the cell-permeable chemical cross-linker DSP (see Experimental Procedures) and lysed in nonreducing Laemmli sample buffer, and the proteins were analyzed by immunoprecipitation. The immunoprecipitated proteins shown in lanes 1, 3, and 5 were derived from control cell lysates (−DSP), while the immunoprecipitated proteins shown in lanes 2, 4, and 6 were derived from cell lysates from DSP-treated cells. The immunoprecipitated proteins were boiled in Laemmli sample buffer containing extra reducing agent (0.25 M DTT and 0.25 M 2-mercaptoethanol) and resolved on 15% SDS−PAGE. Shown is a fluorograph of the gel. Lanes 1 and 2, preimmune serum; lanes 3 and 4, anti-hdj-1p antibody; lanes 5 and 6, anti-Hsp72/73 antibody.

antibodies to Hsp73 (Figure 5B, lane 6) were found to be effective for the capture of the radiolabeled nascent polypeptides.

hdj-1p Destabilizes an Hsp73 Chaperone−Substrate Complex. Having failed to identify either of the two DnaJ homologues in contact with nascent/newly synthesized polypeptides in vivo, we turned our attention to in vitro studies using purified components. Here we examined (i) whether hdj-1p would interact with Hsp73, (ii) whether hdj-1p would act to stimulate the ATPase activity of Hsp73, and finally (iii) whether the presence of hdj-1p would affect the interaction of Hsp73 with an unfolded polypeptide substrate. In the first experiment varying amounts of purified hdj-1p (recombinant protein used at 0.13−5.26 μM) were added to a fixed amount (2.74 μM) of purified Hsp73. The reactions were carried out at 30 °C for 30 min under the following conditions: (1) no added ATP; (2) in the presence of ATP added to a final concentration of 5 mM; or (3) in the presence of 5 mM ATP, followed by the hydrolysis of the ATP via incubation with the enzyme apyrase. The products of each reaction were analyzed by native polyacrylamide gel electrophoresis (and staining of the proteins with Coomassie Blue) to examine possible protein−protein interactions. As shown in Figure 6A, panel a, lane 3, Hsp73 alone migrated as several different species, which likely represent monomer, dimer, and either trimer or tetramer forms of the protein (*44*−*46*). In the case of hdj-1p alone, we were unable to detect the protein migrating into the gel (Figure 6A, panel a, lane 1). Analysis of even higher amounts of the purified protein failed to result in any detectable hdj-1p in the native gels (Figure 6A, panel a, lane 2). hdj-1p does not move toward the anode under the pH conditions (i.e., pH 7.0) used here, presumably because of its very basic isoelectric point.

Addition of substoichometric levels of hdj-1p, relative to Hsp73, had little or no effect on the migration of Hsp73 in the native gel (Figure 6A, panel a, lanes 4 and 5). However, when hdj-1p was added at levels equal to or greater than that of Hsp73, two additional bands were observed very near the top of the gel (Figure 6A, panel a, lanes 6 and 7). The relative levels of these slower migrating species were found to increase when the reactions contained Hsp73, hdj-1p, and ATP, concomitant with a decrease in those faster migrating forms of Hsp73 (Figure 6A, panel b, lanes 5−7). Via Western blotting we confirmed that these slowly migrating species contained Hsp73 (data not shown). Note that the mobility of the Hsp73 monomer was observed to be slightly slower when incubated in the presence of ATP prior to its analysis by electrophoresis (the fastest migrating band in

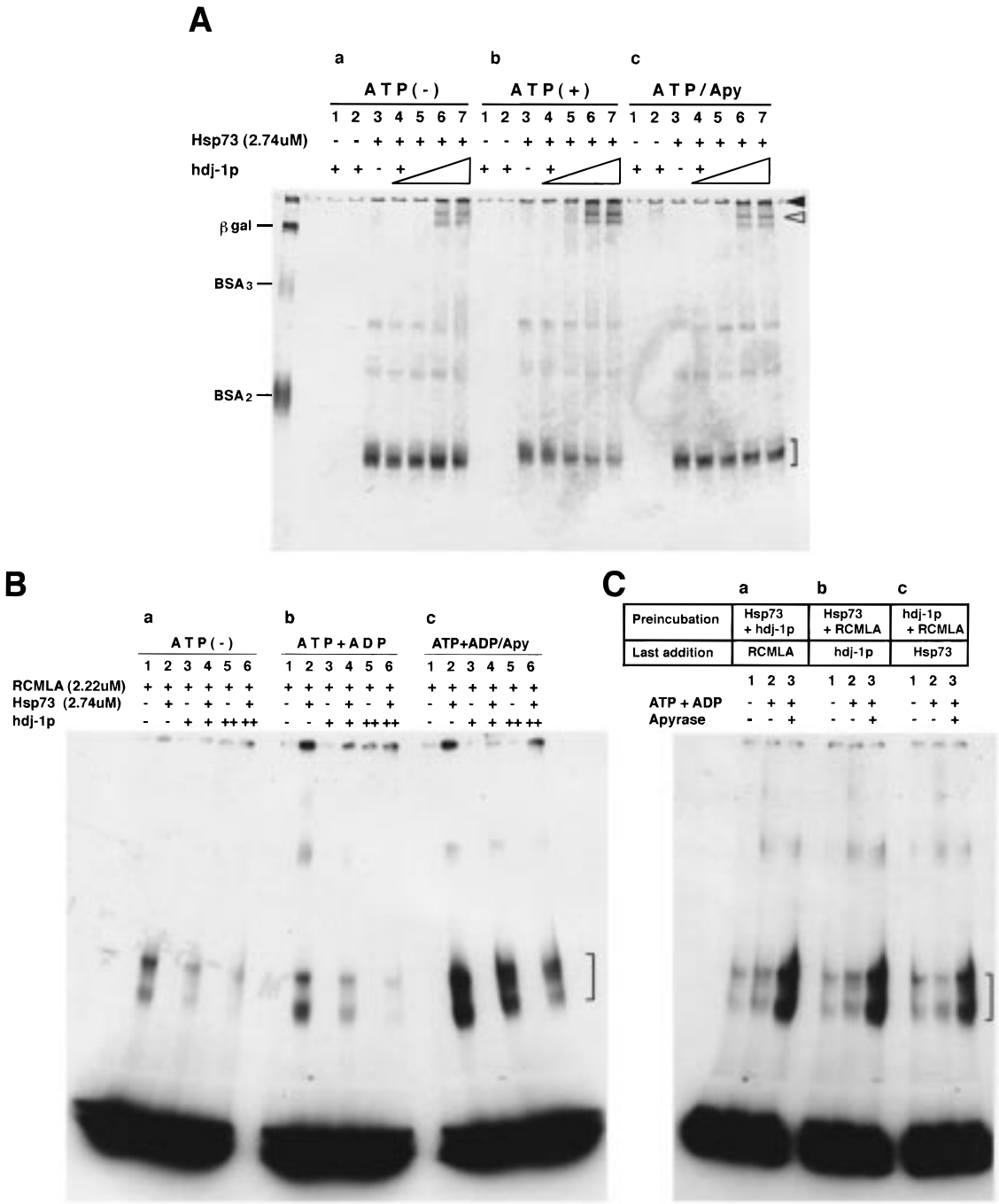

FIGURE 6: Influence of hdj-1p on the native electrophoretic mobility of Hsp73 (A) and complex formation between Hsp73 and RCMLA (B, C). Panel A: Hsp73 or hdj-1p were incubated separately or together in the amounts described below for 30 min at 30 °C without ATP (a, ATP(−)) or with 5 mM ATP (b, ATP(+)) or for 20 min with 5 mM ATP and then 15 min with 25U/mL apyrase (c, ATP/Apy) at 30 °C. The reaction mixtures were analyzed on native PAGE (9/15%) as described in Experimental Procedures. Shown is the gel stained by Coomassie Blue. Lane designations are the same in panels a, b, and c. Lane 1, 0.13 μM hdj-1p; lane 2, 2.6 μM hdj-1p; lane 3, 2.7 μM Hsp73; lane 4, 2.7 μM Hsp73 + 0.13 μM hdj-1p; lane 5, 2.7 μM Hsp73 + 0.26 μM hdj-1p; lane 6, 2.7 μM Hsp73 + 2.6 μM hdj-1p; lane 7, 2.7 μM Hsp73 + 5.3 μM hdj-1p. Arrowheads at the top right of the panels indicate the positions of those species of lower electrophoretic mobility generated by the co-incubation of Hsp73 with hdj-1p. Molecular mass markers are indicated to the left and include (from the top): β-galactosidase (~400 kDa), BSA trimer (~200 kDa), and BSA dimer (~130 kDa). Panel B: Reaction mixtures of [^{125}I]RCMLA (2.2 μM), Hsp73 (2.7 μM), and varying amounts of hdj-1p (below) were incubated for 25 min at 30 °C without (a, ATP(−)) or with 5 mM ATP and 0.1 mM ADP (b, ATP+ADP) or for 20 min with 5 mM ATP and 0.1 mM ADP and then 5 min with apyrase and SBTI (c, ATP+ADP/Apy) at 30 °C. The reaction mixtures were analyzed on native PAGE (9%/15%) as in panel A. Shown is an autoradiograph of the gel. Lane designations are the same in panels a, b, and c. hdj-1p additions included, lane 1, none; lane 2, none; lane 3, 0.26 μM; lane 4, 0.26 μM; lane 5, 2.6 μM; and lane 6, 2.6 μM. Hsp73−RCMLA complexes are indicated by the bracket. Panel C: Two elements of [^{125}I]RCMLA (2.2 μM), Hsp73 (2.7 μM), and hdj-1p (0.26 μM) were preincubated for 10 min at 30 °C without (lane 1) or with 5 mM ATP and 0.1 mM ADP (lanes 2 and 3); then the third element was added, and the samples were further incubated for 15 min (lanes 1 and 2) or for 10 min and then 5 min with apyrase (lane 3) at 30 °C. The reaction mixtures were analyzed by native PAGE, as described in panel B. Shown is an autoradiograph of the gel. In a, Hsp73 and hdj-1p were preincubated and then RCMLA was added; in b, Hsp73 and RCMLA were preincubated and then hdj-1p was added; in c, hdj-1p and RCMLA were preincubated and then Hsp73 was added. Hsp73−RCMLA complexes are indicated by the bracket.

Figure 6A; compare panel b, lane 3, to panel a, lane 3). Finally, in those reactions where ATP was present during the incubation and the nucleotide was then hydrolyzed via the addition of apyrase, the overall levels of oligomerized Hsp73 were reduced, reaching levels similar to that observed when the analysis was performed in the absence of ATP (Figure 6A, panel c). These observations are consistent with previous studies where the yeast DnaJ homologue, ydj-1p, and hdj-1p were reported to stimulate the apparent polymerization of both yeast and bovine brain Hsp73 (*46*, *47*).

Next, we examined whether purified hdj-1p would act to stimulate the relatively weak ATPase activity of the Hsp73 chaperone. Stimulation of hsp70 ATPase activity is one of the most notable functions of members of the DnaJ family (*48*). Hsp73 was incubated with varying amounts of purified hdj-1P in the presence of [α-^{32}P]ATP. After a 15-min reaction at 30 °C, the reaction was quenched by the addition of EDTA and the extent of ATP hydrolysis was determined by thin-layer chromatography. Substoichiometric levels of hdj-1p were sufficient to stimulate Hsp73 ATPase activity 10-fold, similar to what others also have reported (*34*, *49*).

The question of whether hdj-1p could influence the interaction of Hsp73 with an unfolded polypeptide was examined. Similar to what has been reported previously, [^{125}I]RCMLA (reduced and carboxymethylated lactalbumin) served effectively as an unfolded protein target for the Hsp73 chaperone (*4*, *34*, *50*). Analysis by native gel electrophoresis revealed two Hsp73−[^{125}I]RCMLA complexes (Figure 6B), both of which we know contain Hsp73 as determined by Western blotting (data not shown). Either in the presence (Figure 6B, panel b) or in the absence (Figure 6B, panel a) of ATP, complexes between Hsp73 and the iodinated unfolded, protein substrate were observed (note that ATP is not cast into the native gels, nor is it present within the gel running buffer). In either case, addition of purified hdj-1p during the 30-min reactions actually decreased the amount of the Hsp73 chaperone−RCMLA complex detected. When the reactions were repeated in the presence of ATP and ADP, followed by depletion of the ATP/ADP via addition of apyrase, significantly more of the Hsp73−RCMLA complex was observed (Figure 6B, panel c). These results indicate that (i) the presence of ATP appears to favor complex formation; but (ii) in the continued presence of nucleotide, complex dissociation occurs slowly, probably during the early stages of the native gel electrophoresis (before the electrophoresis procedure itself is able to separate the free nucleotide from the protein complex); and (iii) hdj-1p appears to destabilize the Hsp73−RCMLA complexes. [Very similar results were obtained when the analysis was performed using a misfolded staphylococcal nuclease as the substrate for Hsp73 (data not shown).]

We examined whether the order of addition of the different components might have an effect on the overall extent of Hsp73−substrate interaction. For example, others have suggested that DnaJ/hsp40 might act first to bind substrate and subsequently to present the substrate to the hsp70 chaperone (*8*, *26*, *27*) or that hsp70's might require an interaction with a DnaJ homologue prior to their binding with an unfolded protein target (*51*−*53*). As is shown in Figure 6C, the overall extent of complex formation appeared to be independent of the order of addition of the different components.

Previous studies have shown that ATP is required both for the binding of hsp70 to its polypeptide substrate and for its release (*15*, *41*, *51*, *54*−*57*; reviewed in refs *1* and *3*). The results of our experiments shown in Figure 6B,C are consistent with this notion. In the presence of ATP/ADP, maximal complex formation of Hsp73 with the substrate was observed, provided that the nucleotides were hydrolyzed (via apyrase) prior to the native gel electrophoresis. Note, however, that even when excess nucleotides were not hydrolyzed prior to the gel electrophoresis (Figure 6B, panel b), we still were able to detect the Hsp73−RCMLA complex, albeit at levels somewhat lower as compared to those of samples treated with apyrase (e.g., Figure 6B, panel c). How could complex formation persist even in the presence of excess ATP/ADP? A likely explanation here was that the complex being detected had actually re-formed during the early stages of native gel electrophoresis, presumably as ATP was electrophoretically separated from both Hsp73 and the substrate protein. Results of an experiment shown in Figure 7A are consistent with this interpretation. Here, Hsp73, radioiodinated RCMLA, and ATP/ADP all were incubated under the same conditions as were described in Figure 6B, panel b. This time, however, ATP was cast into the native gel as well as added to the running buffer present in the upper gel chamber. Now upon electrophoresis we were unable to detect any Hsp73−RCMLA complex. Thus, in the continued presence of ATP the hsp70 chaperone−polypeptide substrate complex was destabilized.

Another potentially confusing result shown in Figure 6 was our observation that hdj-1p could have effects on both the oligomeric state and the substrate binding affinity of Hsp73 in the absence of *added* nucleotide. For example, addition of high levels of hdj-1p to Hsp73, in the absence of added ATP, resulted in an apparent oligomerization of Hsp73 (Figure 6A, panel a, lanes 6 and 7). Similarly, hdj-1p acted to destabilize the Hsp73−RCMLA complex in the absence of added ATP (Figure 6B, panel a, lanes 4 and 6). Because members of the hsp70 family are routinely purified via ATP affinity chromatography (and released via added ATP), we suspected that our purified Hsp73 protein likely was "contaminated" with low levels of ATP, even though the protein previously had been dialyzed to remove excess nucleotide. Consequently, purified Hsp73 was incubated with apyrase covalently bound to Sepharose beads so as to completely convert any contaminating ATP/ADP to AMP. The immobilized apyrase was then removed by centrifugation and the ATP/ADP-depleted Hsp73 was used in reactions to determine the effect of added hdj-1p upon substrate binding in the presence of a variety of adenine nucleotides. Now in the complete absence of ATP a very low level of Hsp73−substrate complex was formed, either in the absence or in the presence of hdj-1p (Figure 7B, lanes 1 and 2, respectively). Reactions containing 5 mM ATP/0.1 mM ADP were observed to yield more Hsp73−substrate complexes, and addition of hdj-1p decreased the amount of complex detected (Figure 7B, lanes 3 and 4). Maximal Hsp73−substrate complexes were detected when the reaction was carried out in the presence of ATP/ADP and the nucleotides were then hydrolyzed by apyrase prior to the gel electrophoresis (Figure 7B, lane 5). Here again, in the presence of hdj-1p, the overall extent of the Hsp73−RCMLA complex was reduced (Figure 7B, lane 6). Reactions supplemented with only ADP or

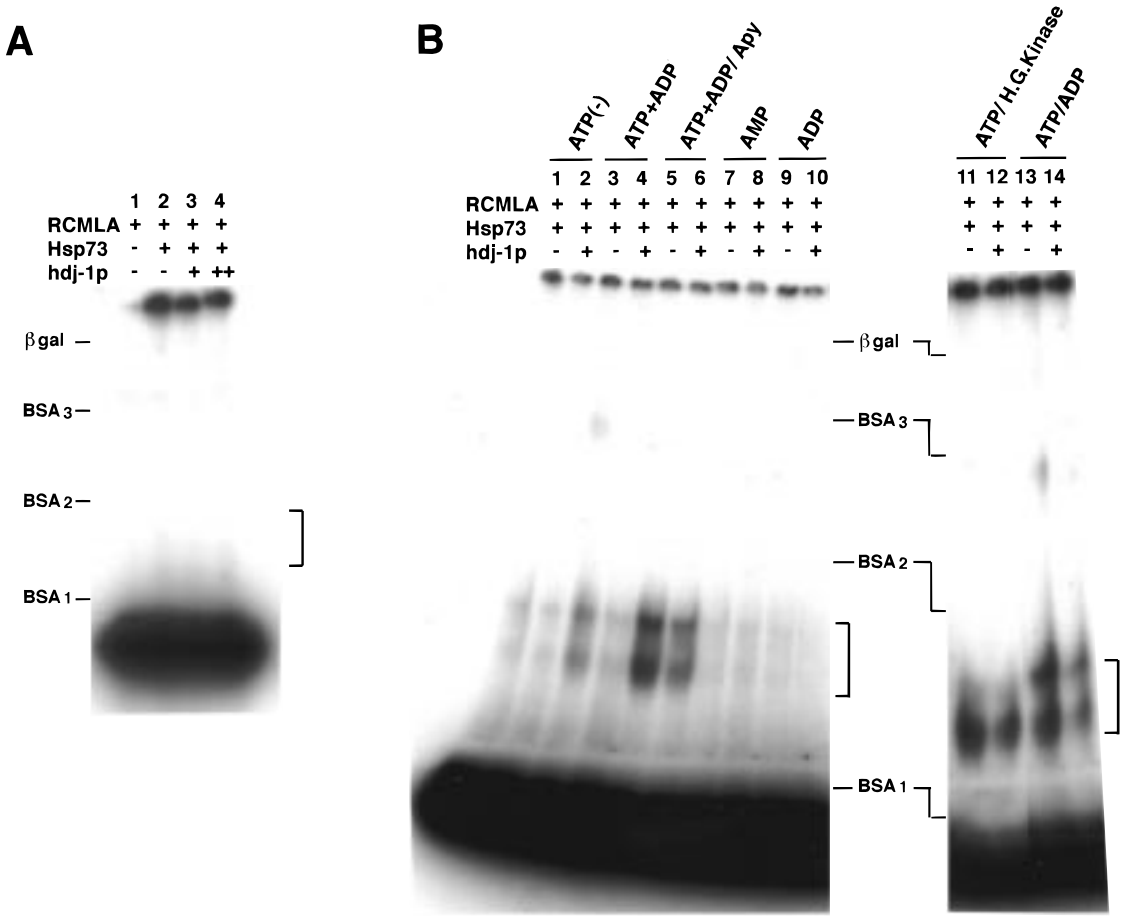

FIGURE 7: Effects of nucleotides and hdj-1p on complex formation between Hsp73 and RCMLA. (A) Reaction mixtures of [^{125}I]RCMLA (1.5 μM), Hsp73 (1.4 μM), and the indicated combinations of hdj-1p (0.18 or 1.8 μM) were incubated for 25 min at 30 °C in the presence of 5 mM ATP and 0.1 mM ADP. The reaction mixtures then were analyzed by native PAGE (9%/15%). Both the gel and the cathode buffer contained 1 mM MgATP. Shown is an autoradiograph of the gel. Lane 1, RCMLA; lane 2, RCMLA + Hsp73; lane 3, RCMLA + Hsp73 + hdj-1p (0.18 μM); lane 4, RCMLA + Hsp73 + hdj-1p (1.8 μM). Hsp73, as detected by Coomassie Blue staining (not shown here), migrated in the gel to the position indicated by the bracket. Molecular mass markers are as indicated in Figure 6A (except BSA1, BSA monomer 68 kDa). (B) Reaction mixtures containing [^{125}I]RCMLA (1.5 μM) and Hsp73 (1.4 μM) in either the presence (even-numbered lanes) or the absence (odd-numbered lanes) of hdj-1p (0.18 μM) were examined as a function of different nucleotide conditions. Here Hsp73 was first incubated with immobilized apyrase to hydrolyze any contaminating ATP or ADP (see Experimental Procedures). The Hsp73 protein was then incubated in the reaction mixtures as indicated. Shown is an autoradiograph of the gel. Lanes 1 and 2, in the absence of ATP (ATP(−)); lanes 3 and 4, in the presence of 5 mM ATP/0.1 mM ADP (ATP+ADP); lanes 5 and 6, in the presence of 5 mM ATP/0.1 mM ADP, followed by incubation with apyrase (ATP+ADP/Apy); lanes 7 and 8, in the presence of 5 mM AMP (AMP); lanes 9 and 10, in the presence of 5 mM ADP (ADP); lanes 11 and 12, in the presence of 5 mM ATP and then with 0.1 mg/mL each yeast hexokinase and *E. coli* glycerol kinase, 100 mM glucose, and glycerol (ATP/H.G.Kinase); lanes 13 and 14, in the presence of 50 uM ATP, followed by addition of 500 μM ADP (ATP/ADP). (Note that two different gels were employed in the analyses.) The position of the Hsp73−RCMLA complexes is indicated by a bracket, and molecular mass markers are the same as in panel A.

AMP yielded no more Hsp73−substrate complex than those reactions performed in the absence of added nucleotide (Figure 7, lanes 7−10).

In contrast to ATP, residual levels of ADP did not appear to affect the Hsp73−RCMLA complex. If the reactions were carried out in the presence of 5 mM ATP and the excess ATP was converted into ADP by incubation with hexose- and glycerol-kinases, Hsp73−RCMLA complex again was observed. The presence of hdj-1p still appeared to reduce somewhat the overall extent of complex formation (Figure 7B, lanes 11 and 12). Note as well that now only the faster migrating Hsp73−RCMLA complex was observed, a result whose significance is not yet clear to us but which could reflect a conformational change of the Hsp73 chaperone as a function of whether ATP or ADP is bound. In a final experiment, we analyzed the effects of very low concentrations of ATP on the formation of the chaperone−substrate complex. Hsp73 and RCMLA, in the presence or absence of hdj-1p, were incubated at the same concentrations as before, but in the presence of only 50 μM ATP. After 20 min of incubation, 500 μM ADP was added, the reactions were allowed to proceed for an additional 5 min, and the samples were then applied to the gel. Under these conditions a significant amount of chaperone−substrate complex was detected, but again the presence of hdj-1p appeared to have a destabilizing effect (Figure 7B, lanes 13 and 14). Note as well that under these conditions (where both ATP and ADP were present) the two Hsp73−RCMLA species again were observed.

DISCUSSION

Results of studies performed in many different laboratories over the past few years have provided considerable support for the idea that protein folding in the cell often requires the participation of accessory components, now referred to as molecular chaperones. One current model describing the

pathway of chaperone-assisted nascent polypeptide folding in the cytosol of mammalian cells involves the sequential cooperation of different members of the molecular chaperone families (*8*). This model has been formulated based upon results of (i) studies of protein folding during in vitro translation experiments (*8, 9*); (ii) studies examining the actions of the purified bacterial molecular chaperones DnaJ, DnaK, and GroEL in the refolding of chemically denatured proteins (*26, 27*); and (iii) analogous experiments determining the role(s) of purified eukaryotic molecular chaperones in the refolding of rhodanese, luciferase, and β-galactosidase (*49, 58*−*60*). In the protein folding pathway proposed to facilitate the folding of nascent firefly luciferase and actin in rabbit reticulocyte lysates, the three different mammalian homologues of the prokaryotic chaperones (hsp40, hsp70, and TRiC (or CCT), respectively) were suggested to function in an ordered and sequential fashion (*8, 9*). Specifically, in this proposed pathway, as the nascent polypeptide chain emerged from the ribosome, with as few as ∼40 amino acids exposed, it would be recognized by and become bound to a member of the DnaJ family, in this case hsp40 (hdj-1p). Contact with the nascent chain by hsp40 then would lead to the subsequent recruitment of Hsp73 to also bind to the substrate, perhaps due to an affinity of hsp70 for hsp 40 (*49, 51, 61*). The stimulation of hsp70's ATPase activity by hsp40 and a triggering of hsp70 into the "closed" conformation (*12, 15, 62*) would result in the stabilization of hsp70 bound to the nascent chain in a ternary complex (hsp40−hsp70−nascent or unfolded chain) (*8, 49*). After some secondary structure had been acquired by the nascent chain, the model then suggests that the nascent polypeptide would be transferred over to the next chaperone, CCT, where subsequent folding of the chain would occur in association with the chaperonin particle (for review, see ref *3*).

There is general agreement within the field that members of the hsp70 family can recognize and bind to unfolded polypeptides, as well as nascent polypeptides undergoing synthesis or translocation into organelles. In the case of the various DnaJ proteins, however, our review of the literature (presented below) reveals a mixed set of observations.

In bacteria, ternary complexes of DnaJ with DnaK (i.e., hsp70 homologue) and a protein substrate (i.e., DnaK−DnaJ−protein substrate) have been reported. For example, DnaK and DnaJ have been implicated in the regulation of transcription factor σ^{32} during the heat shock response (reviewed in ref *1*) and in the initiation of replication of phage λ DNA (*14, 63*). In the case of λ DNA replication, the key intermediate formed contains DnaK, DnaJ, and the λP protein. Sequestration of λP results in the liberation of the DnaB protein (previously bound to λP), thereby allowing it to function as a DNA helicase, a process essential to the DNA replication reaction (*64, 65*). With regard to σ^{32} regulation, DnaJ has been suggested to interact directly with this sigma factor, thereby resulting in the recruitment of DnaK (*66*). Now part of a ternary complex, the sigma factor is prevented from binding to RNA polymerase. It is important to note that in both of these cases (e.g., σ^{32} and λP) the substrate protein being targeted by DnaJ is a mature polypeptide, presumably having already adopted one or more folded states.

Langer et al. was one of the first to report an interaction of bacterial DnaJ with unfolded polypeptides in vitro (*26*). In another study, bacterial DnaJ could be chemically cross-linked to nascent polypeptide chains produced in a rabbit reticulocyte lysate. However, here the purified DnaJ protein was added at a very high molar excess over the newly translated radiolabeled substrate protein (*67*). The association of DnaK and DnaJ with translating ribosomes in *Escheichia coli*, in vivo and in vitro, has been described (*68, 69*). The first step in the chaperone-mediated folding reaction of in vitro translated rhodanese was suggested to be the binding of DnaJ to an N-terminal segment of the nascent chain on the ribosome (*70*). Subsequently, the same group reported that purified bacterial DnaJ could bind directly to a small peptide derived from the N-terminus of bovine rhodanese (*71*).

Via genetic approaches, yeast DnaJ homologues have been implicated as essential cofactors for the family of hsp70 proteins (*72*−*74*). Here, the strongest evidence for a direct interaction of a yeast DnaJ homologue and a polypeptide substrate has been presented in studies of the role(s) of mdj-1p during polypeptide translocation into and folding within mitochondria (*75, 76*). For example, the mdj-1p protein was found to bind to a newly translocated mitochondrial protein during the early stages of its folding. Interestingly, no interaction of mdj-1p with the protein being translocated into the organelle, a process requiring the mitochondrial form of hsp70, was reported. In the case of the endoplasmic reticulum (ER), DnaJ homologues, along with BiP (ER hsp70), are required for the import of proteins from the cytosol (*77*−*80*). Nevertheless, direct interactions between the ER-localized DnaJ homologues and the translocating polypeptide have not been reported (*81*).

With regard to an interaction in vitro between the cytosolic yeast DnaJ homologue, ydj-1p, and denatured protein substrates, mixed results have been reported. For example, in one study ydj-1p was not observed to bind to reduced and carboxymethylated lactalbumin (*50*). In another study ydj-1p was found to prevent the aggregation of chemically unfolded rhodanese that had been diluted from a chaotropic solution (*58*). Here the investigator stated that ydj-1p interacted directly with the unfolded protein substrate. Perhaps most relevant to the results we have presented here are those studies showing that ydj-1p destabilized the complex between hsp70 and the denatured substrate protein RCMLA (*50*). Finally, a very recent report suggested that the amino-terminal region of SV40 T-antigen, which contains a "DnaJ homology box," effected the release of an unfolded substrate polypeptide from the hsp70 chaperone (*82*).

In experimental systems derived from animal cells, there have been two reports that hdj-1p can bind directly to substrate proteins. Frydman et al. (*8*) reported that hsp40 (hdj-1p) was bound to nascent firefly luciferase chains of ∼77 amino acids as synthesis proceeded in the rabbit reticulocyte lysate. This interaction was shown to still occur even when the lysate was previously depleted of hsp70, decreasing the likelihood that the observed hsp40−substrate interaction was solely due to ternary (hsp70−hsp40−substrate) complex formation. In a subsequent study by the same group, hsp40 was observed to coprecipitate with denatured actin after the substrate protein was diluted out of the chaotrope into a rabbit reticulocyte lysate. While hsp70 was found to bind to nascent actin chains as they emerged from the ribosome, the binding of hsp40 to nascent

actin chains was not reported (*9*). In other studies examining protein renaturation, hsp40 was clearly shown to influence the reaction cycle through which hsp70 and other factors acted to renature luciferase (*34*, *49*) and β-galactosidase (*60*, *83*). Here again, there were no data presented demonstrating a direct interaction of hsp40 with the protein undergoing refolding.

Thus, when all the evidence is taken together, there does not appear to be a solid consensus regarding the issue of DnaJ proteins being capable of recognizing and binding to unfolded proteins in vitro, or nascent polypeptides in vivo. Consequently, both here and in a previous study (*33*), we set out to examine the suggestion that different members of the molecular chaperone family interact in a sequential fashion with nascent polypeptides. In the present study we were particularly interested in determining whether DnaJ homologues might bind directly to nascent polypeptides.

The possible interaction of nascent polypeptide chains with the two DnaJ homologues in vivo was examined by a number of independent experimental approaches. First, through use of pulse−chase radiolabeling methods and immunoprecipitation using antibodies to the different chaperones, we were unable to detect nascent polypeptide chains in association with either hdj-1p or hdj-2p (Figure 3A). In contrast, the binding of Hsp73 to nascent chains was very evident. Because many investigators have employed unfolded polypeptides as model substrates to aid in detecting the interaction of the different molecular chaperone family members with substrates in vitro, we applied a similar strategy to trap potential complexes between chaperones and folding intermediates/misfolded polypeptides in vivo. Specifically, we included an amino acid analogue of proline, azetidine, into the cell culture medium during the pulse−chase experiment. In contrast to their interaction with Hsp73, the amino acid analogue containing proteins were not observed to interact with either hdj-1p or hdj-2p with (Figure 3B).

Another method used to generate intermediates in the folding of newly synthesized proteins in vivo has taken advantage of the action of the antibiotic puromycin. This drug induces termination of polypeptide chain synthesis in a random fashion. These prematurely terminated chains have been shown previously to be good substrates for the hsp70 chaperone (*8*, *9*, *32*, *33*, *41*). While we again could detect complexes of Hsp73 with the puromycin-released nascent polypeptides, no interactions of these puromycin-released polypeptides with hdj-1p were observed (Figure 4).

Finally, in experiments where the nascent chains were covalently cross-linked to their nearest neighbors in the cell or in cell lysates, immunoprecipitation using antibodies to hdj-1p again resulted in very little, if any, capture of nascent polypeptides (Figure 5). Cross-linking of the nascent chains to Hsp73, however, was very apparent. Thus, via a number of different experimental approaches, we have been unable to confirm the idea that either of the two cytosolic DnaJ homologues, hdj-1p (hsp40) or hdj-2p, interacts directly with the general population of nascent polypeptides in the cell.

Similarly, using an in vitro approach, Hsp73, but not hsp40, was found to bind a denatured protein substrate, RCMLA. Similar to other reports, we did observe hdj-1p (hsp40) to stimulate the weak ATPase activity of Hsp73 by as much as 10−15-fold (*34*, *49*). When Hsp73 and hsp40 were incubated with the denatured polypeptide in the presence of ATP, hsp40 was found to have a net destabilizing effect upon the hsp70−substrate complex. In agreement with previous studies of bacterial DnaK (for example, see ref *51*), but in contrast to a recent report examining mammalian Hsp73/hsp40 (*49*), we found that maximal complex formation between Hsp73 and substrate required that ATP be present at least transiently and that ADP was insufficient to support complex formation (see Figure 7B).

In summary, our results presented here showed that (1) hdj-1p and 2p did not interact directly with the general population of nascent and newly synthesized polypeptides in mammalian cells; (2) stable complexes between the cytosolic DnaJ homologues and hsp70 were not apparent; (3) loading of Hsp73 onto unfolded polypeptide substrate was maximal in the presence of ATP, provided that ATP levels were subsequently depleted prior to the gel analysis; (4) ATP is required for the release of substrate from Hsp73; and (5) in the presence of ATP, hdj-1p stimulated (or accelerated) release of polypeptide substrate from Hsp73. Thus, upon the basis of these results, we speculate that a tertiary complex involving hdj-1p (i.e., hsp70−hdj-1p−nascent polypeptide) has a very transient existence, if indeed it exists at all in vivo. Because hdj-1p stimulated the hydrolysis of ATP by Hsp73, the DnaJ homologues likely play an important role in activating an ATP-dependent conformational change in the hsp70 chaperones for their subsequent binding to a polypeptide substrate, analogous to what has been proposed for DnaJ effects on DnaK (*12*, *51*). Thus a role for hdj-1p (and other DnaJ homologues) which may reconcile our findings with the results of other published work, we believe, involves J proteins as effectors of a *dynamic interaction cycle* of the hsp70 chaperone with polypeptide substrates. In many cases this interaction may not result in a very long lived hsp70 chaperone−substrate complex. By effecting a rapid transit of substrates through complexes with hsp70, DnaJ-related proteins may advance the rate or efficiency of protein folding. Specifically, promotion of a rapid equilibrium between free and hsp70-bound polypeptide would allow or help ensure that a polypeptide which has not yet achieved its final native folded state to continue to enter into another binding and release cycle involving hsp70, eventually leading to the acquisition of its properly folded state.

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