Cytoplasmic Hsc70 is a multifunctional molecular chaperone. It is hypothesized that accessory proteins are used to specify the diverse chaperone activities of Hsc70. A 16-kDa cytosolic protein (p16) co-purified with Hsc70 obtained from a fish hepatocyte cell line, PLHC-1. Hsc70 also co-immunoprecipitated with p16 from PLHC-1 cells and fish liver. p16 was identified as a member of the Nm23/nucleoside diphosphate (NDP) kinase family based on its amino acid sequence similarity, NDP kinase activity, and recognition by anti-human NDP kinase-A antibody. This antibody also co-immunoprecipitated Hsc70 and NDP kinase from human HepG2 cells. p16 monomerized Hsc70 and released Hsc70 from pigeon cytochrome c peptide (Pc) but not from FYQLALT, a peptide specifically designed for high affinity binding. Therefore, p16 may modulate Hsc70 function by maintaining Hsc70 in a monomeric state and by dissociating unfolded proteins from Hsc70 either through protein-protein interactions or by supplying ATP indirectly through phosphate transfer. p16 did not affect basal or unfolded protein-stimulated ATPase activity of bovine brain Hsc70 using in vitro assays. Interestingly, bovine liver NDP kinase did not dissociate the Hsc70-Pc complex. In addition, two nonconservative amino acid substitutions were found near the amino terminus of p16. Therefore, p16 may be a unique Nm23/NDP kinase that functions as an accessory protein for cytosolic Hsc70 in eukaryotes.

Organisms respond to a variety of chemical and physiological stresses by rapidly synthesizing a group of conserved polypeptides known as heat shock or stress proteins. The induction of these stress proteins is a defense against proteotoxicity, a term used to describe damage to proteins caused by diverse stressors (1). Interestingly, in addition to their function in stressed cells, some members of these stress protein families are normally abundant in nonstressed cells, such as the constitutively expressed 70-kilodalton (kDa) heat shock cognate protein (Hsc70). Hsc70 functions as a molecular chaperone to maintain protein homeostasis (protein folding, translocation, assembly, disassembly, and degradation) in cells under normal conditions as well as during stress (reviewed in Refs. 2–4). Hsc70 was originally characterized as an uncoating ATPase that dissociates clathrin triskelions from clathrin-coated vesicles (5–7). It maintains the translocation-competent state of proteins destined for endoplasmic reticula and mitochondria (8–10) and transiently interacts with nascent polypeptides during translation (11, 12). Hsc70 is a crucial component in targeting proteins to lysosomes for degradation (13, 14) and in importing cytoplasmic proteins into the nucleus (15–17). Hsc70 cooperates in Tric-mediated folding (18) and may be involved in targeting proteins to the ubiquitin/proteasome machinery for degradation (19, 20). In cooperation with Hsp90, Hsc70 is involved in steroid receptor signaling (reviewed in Refs. 21–23). Hsc70 also binds to retinoblastoma protein (24) and to a mutant form of tumor suppressor protein p53 (25). In addition, Hsc70 suppresses protein aggregation (26) and reactivates heat-denatured proteins (27). Hsc70 may also transiently bind to monomeric heat shock transcription factor to keep it in an inactive state. (28, 29).

Hsc70 participates in these diverse cellular processes through the binding and release of protein substrates at the expense of ATP hydrolysis. Hsc70 recognizes short extended peptide sequence motifs enriched in either aromatic/hydrophobic or hydrophobic/basic residues (30, 31). Flexibility in the Hsc70 peptide binding domains can only explain part of the diverse functions of Hsc70. Recent research has suggested that specific accessory proteins may help to determine the diverse chaperoning activities of Hsc70 (reviewed in Ref. 32). For example, the accessory proteins DnaJ and GrpE cooperate with DnaK in Escherichia coli in the replication of a phage (33) and P1 plasmid (34), in the negative autoregulation of the heat shock response (35–37), and in the reactivation of heat-inactivated RNA polymerase (27) and firefly luciferase (38). These two accessory proteins have different actions on DnaK. DnaJ can present certain substrates to DnaK and stimulate the ATPase activity of DnaK while GrpE seems to act as a nucleotide exchange factor (34, 39).

Although a eukaryotic GrpE homolog has only been found in mitochondria (40–43), a family of DnaJ homologs has been discovered based on the similarity of their N-terminal J-domains (1–70 amino acid residues; see Ref. 44). However, their limited sequence similarity besides the J-domain suggests that the function of each DnaJ homolog may have become specialized (45). For example, in yeast, the cytosolic DnaJ homolog Sis1 binds to Hsc70 during translation while Ydj1, another DnaJ homolog, is thought to target Hsc70 bound substrates to mitochondria and endoplasmic reticula (46, 47). Furthermore, overexpression of Ydj1 cannot rescue Sis1 deletion phenotypes, suggesting that Sis1 has a more specialized function (48). In addition to DnaJ and GrpE homologs, there appear to be a number of other cofactors that are involved in specific Hsc70

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The abbreviations used are: Hsc70, 70-kDa heat shock cognate protein; NDP, nucleoside diphosphate; Pc, pigeon cytochrome c peptide; CMF-PBS, calcium- and magnesium-free phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; apo c, apocytochrome c; TLC, thin layer chromatography.

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chaperone functions. For example, a 100-kDa protein, auxilin, is required for Hsc70 to coat clathrin-coated vesicles (49–51). Recently, Hartl and co-workers (52) isolated a 41-kDa Hsc70-interacting protein, Hip, using the yeast two-hybrid system. Hip may modulate the interaction of Hsc70 with different substrates through the stabilization of the ADP-bound state of Hsc70.

In further support of the hypothesis that multiple accessory proteins are required for diverse chaperoning functions of Hsc70, we report here the isolation of a new cytosolic accessory protein for eukaryotic Hsc70. This 16-kDa protein (p16) monopolizes Hsc70 and dissociates Hsc70 from peptide substrate; it is a member of Nm23/nucleoside diphosphate kinase family.**

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### EXPERIMENTAL PROCEDURES

**Cell Culture—*P. lucida* hepato-cellular carcinoma (PLHC-1) cells were isolated from a 7,12-dimethylbenz[a]anthracene-induced hepato-cellular carcinoma of a desert topminnow *P. lucida* (53, 54). PLHC-1 cells were grown in Eagle’s minimal essential medium with Earle’s buffer (Life Technologies, Inc.), pH 7.2, supplemented with 10% (v/v) calf serum (J. R. H. BioSci) and 4 mM -glutamine in a 30°C humidified incubator containing 5% CO2. No antibiotics were used. Culture media were mycophenolate-free, as described previously. Human hepato-cellular carcinoma (HepG2 cells, ATCC HB 8065) were grown in CO2-independent medium (HepG2 cells, ATCC HB 8065) were grown in CO2-independent medium (Life Technologies, Inc.) with 5% fetal bovine serum (Hyclone Laboratories) at 37°C. Confluent PLHC-1 cells or HepG2 cells were dissociated with 0.05% (v/v) trypsin and 0.5 mM EDTA in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS), and the cells were harvested by centrifugation at 10 × g for 5 min. Harvested cells were washed once in serum-containing medium to inactivate the trypsin and then three times in CMF-PBS. Then the cells were immediately frozen in liquid nitrogen and stored at −70°C. Usually four 25-cm2 confluent PLHC-1 cultures yielded 1 g of cells.**

### Purification of p16 and Hsc70—Bovine Hsc70 was purified according to Sadis and Hightower (56). p16 was purified by lysing PLHC-1 cells in 5 volumes of lysis buffer (11 mM Tris-Cl, pH 8.5, 100 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 150 mM β-mercaptoethanol, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) for 10 min. Then the cell lysate was clarified by ultracentrifugation at 70,000 × g for 1.5 h and dialyzed overnight against 40–50 volumes of buffer C (20 mM Hepes-KOH, pH 7.0, 25 mM KCl, 10 mM (NH4)2SO4, 2 mM EDTA, 0.1 mM NaF, and 0.1 mM magnesium acetate) and subjected to ATPase chromatography (linked through C-8, Sigma). The ATP-agarose was washed with 0.5 M KCl to remove nonspecific binding proteins, and then the bound proteins were eluted with 1 bed volume of 1 mM ATP (to remove the ATPase activity) was determined by measuring the release of radioactive inorganic phosphate (32P) from [γ-32P]ATP (57).

**Internal Amino Acid Sequencing—** Purified p16 was cleaved by endoprotease-Arg-C to generate peptide fragments for internal amino acid sequencing. Two of the HPLC-purified peptide fragments were sequenced (Protein Structural Laboratory, University of California at Davis). The resulting sequences were used to search for similarity in the GenBank and SwissProt data bases.

**NDP Kinase Activity of p16—** NDP kinase activity of purified p16 was determined by thin layer chromatography (TLC) using alkaline phosphatase-conjugated secondary antibody (Sigma) with 4-chloro-3-indolyl phosphate/nitroblue tetrazolium as the substrate. The gels were then fixed in 30% methanol, 10% acetic acid and silver stained. An affinity purified rabbit polyclonal antibody raised against human Hsc70-A (Oncor, Gaithersburg, MD) was used to identify p16. Hsc70 and Hsp70 were detected by using a monoclonal antibody (clone 3a3) raised against human Hsp70 (Affinity BioReagents, Neshanic Station, NJ). Antibody binding was visualized by a colorimetric method using alkaline phosphatase-conjugated secondary antibody (Sigma) with 4-chloro-3-indolyl phosphate/nitroblue tetrazolium as the substrate. The color development was stopped by adding 20 mM EDTA.

Co-immunoprecipitation of p16 and Hsc70 from PLHC-1 Cells—All procedures were performed at 4°C. PLHC-1 cells, HepG2 cells, or *P. lucida* liver tissue were resuspended in 3 volumes of cold hypotonic buffer (1.5 mM MgCl2, 5 mM KCl, 10 mM (NH4)2SO4, 2 mM EDTA, 0.1 mM NaF, and 0.1 mM magnesium acetate) and subjected to ATPase chromatography (linked through C-8, Sigma). The ATP-agarose was washed with 0.5 M KCl to remove nonspecific binding proteins, and then the bound proteins were eluted with 1 bed volume of 1 mM ATP (to remove the ATPase activity) was determined by measuring the release of radioactive inorganic phosphate (32P) from [γ-32P]ATP (57).

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To purify p16, a narrow salt gradient (0.05–0.15 M KCl) was used. Induction of Hsp70 and p16 by Cadmium Ions—Confluent PLHC-1 cells were exposed to 0, 0.5, 5.0, and 7.5 mg/liter heavy metal cadmium ions (CdCl₂) for 72 h to maximize protein accumulation, and then the cells were solubilized in 200 μl of SDS-PAGE sample buffer. Actin was used as an internal control for equal amounts of protein loading on SDS-PAGE. The changes in Hsc70 and Hsp70 combined (Hsp70s) and p16 protein levels were detected by SDS-PAGE and immunoblotting using 3a3 and anti-NDP kinase-A antibody, respectively.

RESULTS

Co-purification of p16 and Hsc70—Our initial goal was to isolate Hsc70 from PLHC-1 cells using conventional chromatography. However, a 16-kDa protein, as determined by Tricine-SDS-PAGE (data not shown), co-purified with Hsc70 using three different types of chromatography, ATP-agarose, anion exchange, and gel filtration chromatography (Fig. 1). p16 and actin (identified by molecular mass and binding to DNA-Sepharose) were co-eluted with Hsc70 from the ATP-agarose column (Fig. 1A) using 5 mM ATP. When the Hsc70-enriched fractions (8–21) from the ATP-agarose were pooled, dialyzed, and then fractionated by Mono Q anion exchange chromatography (Fig. 1B), actin (fractions 19–21) was well separated from the majority of Hsc70 (fractions 14–16) while p16 (fractions 13–14) still overlapped with the leading edge of Hsc70. Because of the differences in their polypeptide molecular weights, we attempted to separate Hsc70 and p16 using Superose-12 gel filtration chromatography (Fig. 1C). Fractions 11–12 represented the protein peak of Hsc70 monomer, determined using molecular weight standards. Interestingly, p16 (fractions 12–14) still partially overlapped with Hsc70 monomer. Because this small protein co-purified with Hsc70, we decided to investigate the possibility that p16 is an Hsc70-associated protein. To purify p16, a narrow salt gradient (0.05–0.15 M KCl) was used to separate a portion of p16 from Hsc70 in the Mono Q column. These fractions containing only p16 were used for assays.

Effect of p16 on Bovine Hsc70, Its Peptide Complexes and ATPase Activity in Vitro—If the co-purification of p16 and Hsc70 is not a coincidence, then p16 could either be an accessory protein or protein substrate of Hsc70. Since protein substrates form complexes with Hsc70 and stimulate Hsc70 intrinsic ATPase activity and since accessory proteins, such as DnaJ and GrpE, modulate Hsc70 peptide/protein substrate binding and ATPase activity, functional assays were used to address whether p16 can affect 1) nondenaturing gel mobility, 2) peptide substrate binding, and 3) ATPase activity of Hsc70.

In the presence of p16, the relative amount of Hsc70 dimers and trimers decreased and the amount of Hsc70 monomer increased (Fig. 2, lanes 1 and 2). The monomeric, dimeric, and trimeric Hsc70 protein bands in nondenaturing gel were estimated to be 70-, 140-, and 210-kDa proteins, respectively, using Superose-12 gel filtration (data not shown). In addition, the p16 protein band was more intensely stained by silver after incubation with Hsc70 (Fig. 2, lanes 2 and 3). There are two possible explanations for this effect. First, p16 may exist as high molecular weight oligomers that cannot migrate into the gel. In the presence of Hsc70, these oligomers may break down allowing more p16 to migrate into the gel. Second, p16 may undergo a conformational change in the presence of Hsc70 resulting in more intense silver staining.

Next we used synthetic peptides to investigate whether p16 can affect Hsc70-substrate interaction. Both P₃ and FYQLALT (30, 31, 58) bind strongly to Hsc70 and stimulate Hsc70 ATPase activity. The Hsc70-P₃ complex migrated slower than the Hsc70 monomer (Fig. 3, lane 2) in the nondenaturing gel. The migration of Hsc70-P₃ complex in nondenaturing gel was identified by the presence of radioactivity using tritium-labeled P₃ as a substrate (data not shown). In the presence of p16, Hsc70 was dissociated from P₃ (Fig. 3, lane 3). Therefore, these data suggested that p16 indeed has a specific functional relationship with Hsc70. Interestingly, the effect of p16 on the Hsc70-FYQLALT complex was very different. Hsc70-FYQLALT migrated faster than the Hsc70 monomer (Fig. 3, lane 4) and p16 did not change the mobility of Hsc70-FYQLALT complexes (Fig. 3, lane 5). However, the mobility of p16 itself was slowed reproducibly in the presence of both Hsc70 and FYQLALT (Fig. 3, lane 5) but not with FYQLALT alone (Fig. 3, lane 7). These data suggest that p16 undergoes a shape change in the presence of Hsc70-FYQLALT complexes. It may be able to bind to FYQLALT when presented by Hsc70 or p16 may interact dif-
Fig. 3. Effect of p16 on Hsc70-peptide complexes. The same amount of p16 was incubated with each preformed Hsc70-peptide complex, and then the proteins were analyzed by nondenaturing gel electrophoresis and silver staining. Lane 1, Hsc70 with p16; lane 2, Hsc70-Pc complex (indicated by filled triangle); lane 3, Hsc70-Pc complex with p16; lane 4, Hsc70-FYQLALT complex; lane 5, Hsc70-FYQLALT complex with p16 (the shift of p16 mobility is indicated by filled circle); lane 6, p16 with Pe; and lane 7, p16 with FYQLALT.

Fig. 4. Effect of p16 on intrinsic and apo c-stimulated ATPase activity of Hsc70. Intrinsic and apo c-stimulated ATPase activity of Hsc70 was determined by measuring the release of radioactive inorganic phosphate (32Pi) from [γ-32P]ATP. Shown are apo c-stimulated ATPase activity, Hsc70 and apo c were incubated with p16 (solid circles) or without p16 (open circles); intrinsic ATPase activity, Hsc70 was incubated with p16 (solid squares) or without p16 (open squares); control, p16 was incubated with apo c (solid triangles) or without apo c (open triangles).

Fig. 5. Amino acid sequence similarity of p16 and human Nm23/NDP kinase. p16 was cleaved by the endoproteinase Arg-C, and two of the resulting peptide fragments were sequenced. They showed similarity and identity to human Nm23/NDP kinase (59). Identical amino acid residues are indicated by lines.

Fig. 6. NDP kinase activity of p16. p16 was incubated with ATP and GDP in the absence (lane 1) or presence (lane 2) of 75 mM EDTA; the nucleotides were then separated using thin layer chromatography and visualized under UV light.

Fig. 7. Co-immunoprecipitation of p16 and Hsc70 from PLHC-1 cell lysate. As shown in Fig. 8, lane 1, when anti-NDP kinase-A antibody was used for co-immunoprecipitation of p16 and Hsc70, the antibody bound only to p16. Therefore, based on its amino acid sequence similarity, NDP kinase activity, and recognition by anti-NDP kinase antibody, we have confirmed that p16 is an NDP kinase.

We then attempted to confirm the identity of p16 as an NDP kinase by immunoblot analysis using a commercially available anti-NDP kinase antibody. Fig. 7 shows that PLHC-1 cell lysate (lanes 2 and 4) and purified p16 (lanes 3 and 5) were analyzed by SDS-PAGE and silver staining (A) or immunoblot analysis (B). An affinity purified rabbit polyclonal antibody raised against human NDP kinase-A was incubated in both PLHC-1 cell lysate and purified p16. Fig. 7B shows that the antibody bound only to p16. Therefore, based on its amino acid sequence similarity, NDP kinase activity, and recognition by anti-NDP kinase antibody, we have confirmed that p16 is an NDP kinase.

Co-immunoprecipitation of p16 and Hsc70 from PLHC-1 Cells—The polyclonal anti-NDP kinase-A antibody was then used for co-immunoprecipitation of p16/Hsc70 complexes from PLHC-1 cell lysate. As shown in Fig. 8, lane 1, when anti-NDP kinase-A antibody was used for immunoprecipitation, Hsc70 was found associated with the p16-antibody immune complex. In the control immunoprecipitation, neither p16 nor Hsc70 was
Fig. 7. Immunoblot analysis of p16 using a polyclonal antibody raised against human NDP kinase-A. Samples from PLHC-1 cell lysate and purified p16 were analyzed by SDS-PAGE and silver staining (A) or transferred to polyvinylidene difluoride membrane for immunoblot analysis using anti-human NDP kinase-A antibody (B). Lane 1, molecular weight markers; lanes 2 and 4, PLHC-1 cell lysate; and lanes 3 and 5, p16.

Fig. 8. Co-immunoprecipitation of p16 and Hsc70 from PLHC-1 and HepG2 cell lysates using anti-human NDP kinase-A antibody. Anti-human NDP kinase-A antibody (lanes 1 and 3) or control antibody (lanes 2 and 4) was added to PLHC-1 or HepG2 cell lysate for immunoprecipitation. Hsc70 (top panel) and p16/NDP kinase (bottom panel) were detected by SDS-PAGE and immunoblotting using 3a3 and anti-human NDP kinase-A antibodies, respectively. The immunoglobulin (Ig) light chain (LC) and heavy chain (HC) staining resulted from a cross-reaction of the secondary antibody used for immunoblotting.

found (Fig. 8, lane 2). Similar results were obtained using *P. lucida* liver tissue (data not shown). To prove that the association of p16/NDP kinase and Hsc70 exists in other vertebrate cells, human hepatocellular carcinoma HepG2 cells were used for immunoprecipitation. As shown in Fig. 8, bottom panel, lane 3, human NDP kinase A and B, with a molecular mass of approximately 18 kDa each, were identified using anti-NDP kinase-A antibody, and Hsc70 was associated with NDP kinase-antibody immune complex (Fig. 8, top panel, lane 3). Again, neither NDP kinase nor Hsc70 was found in the control immunoprecipitation (Fig. 8, lane 4). Therefore, Hsc70 indeed bound to p16/NDP kinase and co-immunoprecipitated from PLHC-1 and HepG2 cell lysate as well as *P. lucida* liver cells lysate with the anti-NDP kinase-A antibody. Also, p16 appears to be a cytoplasmic protein rather than a membrane-bound protein since it can be easily released from cells using hypotonic lysis. Next, we used glutaraldehyde to cross-link purified Hsc70 and p16 and then identified the cross-linked product by SDS-PAGE and immunoblotting using 3a3 and anti-NDP kinase antibody. When a mixture of Hsc70 and p16 was treated with glutaraldehyde, the pattern of cross-linked proteins was altered when compared with their individual self cross-linking patterns. A new high molecular weight cross-linked protein band was identified by anti-NDP kinase antibody (data not shown). However, the interpretation of this new protein band as a potential Hsc70-p16 cross-linked product was complicated by the extensive self cross-linking of Hsc70 in the same high molecular weight region (data not shown).

**ATP, but Not Bovine NDP Kinase Dissociated Hsc70 from Pc**—Since ATP can monomerize Hsc70 (61, 62) and ATP binding causes substrate release from both Hsc70 and DnaK in the presence of potassium ions (63), p16 may affect Hsc70 functions by providing ATP through its NDP kinase activity. We first examined the effect of ATP on the Hsc70-Pc complex. As expected, ATP effectively released Hsc70 from Pc (Fig. 9, lane 1). Then we investigated whether mammalian NDP kinases can interact with Hsc70. The commercially available NDP kinase purified from bovine liver contained two polypeptides with approximate molecular masses of 18 and 19 kDa, respectively (data not shown); and it was able to transfer γ-phosphate from ATP to GTP (data not shown). However, bovine NDP kinase not only did not dissociate Hsc70 from Pc (Fig. 9, lane 2), but it also migrated much slower than p16 during non-denaturing gel electrophoresis (Fig. 9, lane 3). Since the known oligomeric structures of NDP kinases (64) are either hexamers (*human, Drosophila, and Dictyostelium*) or tetramers (rat, yeast, and *Mycobacterium*), it is possible that p16 and bovine NDP kinase migrated as tetramer and hexamer, respectively.

**p16 Is a Stress-inducible Protein**—The protein levels of p16 increased with increasing concentrations of cadmium ions (Fig. 10 bottom panel, lanes 1–4), a stressor that can induce cellular stress responses. Therefore, p16 is a stress-inducible protein. In addition, p16 showed cellular stress response to cadmium ions at lower concentrations with maximal protein accumulation at 5.0 mg CdCl₂/liter (Fig. 10, bottom panel, lane 3) compared with that of Hsc70/Hsp70 at 7.5 mg CdCl₂/liter (Fig. 10, top panel, lane 4).

**Hsc70 Is Not Phosphorylated by p16**—Next, we used phosphorylation assay and nondenaturing gel electrophoresis to investigate whether p16 can phosphorylate Hsc70 by transferring ATP to GTP (data not shown); and it was able to transfer γ-phosphate from ATP to GTP (data not shown). However, bovine NDP kinase not only did not dissociate Hsc70 from Pc (Fig. 10, lane 1), but it also migrated much slower than p16 during non-denaturing gel electrophoresis (Fig. 10, lane 2). Since the known oligomeric structures of NDP kinases (64) are either hexamers (*human, Drosophila, and Dictyostelium*) or tetramers (rat, yeast, and *Mycobacterium*), it is possible that p16 and bovine NDP kinase migrated as tetramer and hexamer, respectively.

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kinase (nucleotide binding and its native oligomeric state) and may have been fortuitous. However, subsequent analyses taken together make a strong case that p16 and Hsc70 interact: 1) Hsc70 co-immunoprecipitates with p16 from PLHC-1 cells, 2) p16 monomerizes Hsc70, and 3) p16 dissociates Hsc70 from Pc. Since unfolded or denatured proteins can bind to and stimulate intrinsic ATPase activity of Hsc70, the association between p16 and Hsc70 could result from the denaturation of p16 during purification. However, our data exclude this possibility because p16, like Hip, does not stimulate Hsc70 ATPase activity; and purified p16 has NDP kinase activity, i.e. it is an enzymatically active protein. Therefore, p16 appears to be an accessory protein that can modulate the oligomeric state of Hsc70 and dissociate unfolded proteins from Hsc70 in the absence of exogenous ATP. In further support of this hypothesis, our data show that p16 is a stress-inducible protein; it is possible that this association may assist Hsc70 and/or Hsp70 in protecting cells from stress-induced damage.

How might p16 modulate Hsc70 chaperone functions? p16 may be involved in maintaining the active monomeric pool of Hsc70 inside cells. It has been shown that purified Hsc70 exists as monomers, dimers, and trimers (5, 61, 65). A current model suggests that the oligomers are the storage/sequestration state of Hsc70/DnaK. Greene and colleagues showed that in the presence of ATP, Ydj1, a DnaJ homolog isolated from yeast, can induce oligomerization of bovine Hsc70 and yeast cytosolic Hsc70 homolog, Ssa1 (72). These data strongly suggest that

besides regulating Hsc70 ATPase and substrate binding activities, accessory proteins also play a very important role in modulating the oligomeric states of Hsc70. By maintaining a monomeric pool of Hsc70, p16 may allow Poeciliopsis to adapt to rapid temperature fluctuations in the desert environment; these fish commonly experience substantial temperature extremes (4–40 °C) and can be exposed to a 22 °C temperature differential in the course of a single day (73, 74). It is possible that p16 may need to release Hsc70 from substrate to allow thermal inactivation of Hsc70 during cooling and to monomerize oligomers rapidly during heating to activate Hsc70. p16 also helps dissociate Hsc70 from Pp but not from FYQ-LALT, suggesting that p16 may modulate specific Hsc70 chaperoning activities. What chaperoning functions of Hsc70 may involve accessory proteins like p16? For example, the Hsc70/clathrin complex has been reported to lock into a stable complex after release from a coated vesicle (58), and therefore, it is possible that accessory proteins like p16 help dissociate Hsc70 from clathrin to recycle both proteins. The efficient dissociation of the Hip/Hsc70-substrate complex may also require p16-like proteins rather than exchange of ADP for ATP (52). In yeast, Ssa2 (cytosolic Hsc70 homolog) and Ydj1 form a stable complex with denatured rhodanese to prevent aggregation (26); accessory proteins like p16 may then help release the denatured rhodanese from Ssa2 and Ydj1 for refolding or degradation. It is also possible that p16-like proteins can function as the cytosolic factors that dissociate Hsc70 from presecretory proteins (75) or mitochondrial precursor-Hsc70 complexes (10). Since the dissociation of Hsc70-Pc complex by p16 did not require exogenous ATP, it supports the idea that Hsc70 does not always require ATP for substrate release. For example, the refolding of heat-denatured topoisomerase I by purified Hsc70 in vitro does not require exogenous ATP (76), and the Hsc70-dependent pathway for mitochondrial import does not require exogenous ATP (77). Therefore, p16 may be a specific regulator for certain Hsc70 chaperone functions that require dissociation of stable Hsc70-substrate complexes at the right time or right place.

Although GrpE can monomerize DnaK (71), our data did not suggest that p16 is a GrpE homolog. First, p16 does not stimulate the nucleotide exchange of Hsc70 (data not shown). Second, GrpE by itself does not release substrate from DnaK without added nucleotides. Finally, unlike GrpE (78), p16 does not appear to form a stable complex with Hsc70 in our non-denaturing gel assay conditions. This transient interaction may explain why p16 was not identified before in Hsc70 complexes. When compared with eDnaJ, p16 appears to have opposite effects on Hsc70 functions. eDnaJ induces oligomerization of Hsc70, presents some substrates to Hsc70 or dissociates some substrates from Hsc70 in the presence of ATP, and stimulates Hsc70 ATPase activity. However, p16 monomerizes Hsc70, dissociates Hsc70 from certain substrates in the absence of ATP, and does not stimulate Hsc70 ATPase activity. Therefore, p16 appears to be a unique accessory protein that may counter the actions of eDnaJ on Hsc70.

p16 may mimic the effect of ATP by providing ATP to Hsc70 indirectly through phosphate transfer. However, our preliminary data suggested no direct phosphorylation of Hsc70-bound ADP by p16 using [γ-32P]ATP (see "Results"). Also, p16 did not stimulate Hsc70 ATPase activity, arguing against this mechanism. Since the active site for phosphate transfer on all NDP kinases involves a histidine residue (64), use of site-directed mutagenesis to replace the active site histidine residue on p16 may answer whether the action of p16 on Hsc70 is through its NDP kinase activity. Interestingly, NDP kinases have been implicated in Drosophila embryo development (79, 80), binding

**Fig. 10. The induction of p16 protein by cadmium ions.** PLHC-1 cells were exposed to increasing concentrations of cadmium ions (lanes 1–4) for 72 h, and the cells were solubilized by adding SDS-PAGE sample buffer. The protein levels of both Hsc70 and Hsp70 (Hsp70s, top panel) and p16 (bottom panel) were then analyzed by SDS-PAGE and immunoblotting using 3a3 and anti-human NDP kinase-A antibodies, respectively.

| Hsp70s | 0 | 0.5 | 5.0 | 7.5 mg/L Cd |
|-------|---|-----|-----|-------------|
| p16   | 1 | 2   | 3   | 4           |
Cromoglycate is a widely used antiasthmatic drug that is structurally related to quercetin, a potent NDP kinase inhibitor in vitro (IC$_{50}$ is 28.6 mm; see Ref. 96). More importantly, quercetin down-regulates the heat shock response in human HeLa cells by reducing both HSF1 phosphorylation and protein levels. As a result, HSF1 heat shock element complex declines (97).

The discovery of p16 as a new cytoplasmic accessory protein for Hsc70 supports the hypothesis that a variety of accessory proteins (eDNAJ, auxilin, Hip, and p16/Nm23/NDP kinase) are required to support the diverse chaperoning functions of Hsc70. These diverse accessory protein-chaperone interactions may allow the cells to regulate different chaperoning functions of Hsc70 more efficiently by controlling the individual accessory proteins. Roles for p16 in the Hsc70 reaction cycle are proposed in Fig. 11. In this model, Hsc70 initiates interaction with substrates in its ATP-bound state, which has a high on rate for substrate. In contrast, the ADP-bound Hsc70 has a low on rate for substrate and, therefore, plays a less important role in initiation of substrate binding. After binding to a substrate, the ATP-bound Hsc70 undergoes a conformational change. Since the ATP-bound Hsc70 is characterized by rapid substrate association and dissociation kinetics, a stable Hsc70-substrate complex will form only after ATP hydrolysis. After nucleotide exchange, Hsc70 returns to its ATP-bound form, and the substrate is subsequently released. Accessory proteins modulate different steps in the reaction cycle. DnaJ-like protein modulates the binding of substrates as well as stimulates ATP hydrolysis, while Hip stabilizes the Hsc70-ADP-substrate complex. Since ATP/ADP exchange is slow and no GrpE homologs have been found in the cytoplasm, some substrates would be released very slowly without help from accessory proteins. However, in the presence of p16, substrate can also be released from Hsc70-ADP, and the subsequent ATP/ADP exchange then converts Hsc70 to the form most accessible for substrate binding.

p16 also monomerizes Hsc70 more efficiently by controlling the individual accessory proteins. Roles for p16 in the Hsc70 reaction cycle are proposed in Fig. 11. In this model, Hsc70 initiates interaction with substrates in its ATP-bound state, which has a high on rate for substrate. In contrast, the ADP-bound Hsc70 has a low on rate for substrate and, therefore, plays a less important role in initiation of substrate binding. After binding to a substrate, the ATP-bound Hsc70 undergoes a conformational change. Since the ATP-bound Hsc70 is characterized by rapid substrate association and dissociation kinetics, a stable Hsc70-substrate complex will form only after ATP hydrolysis. After nucleotide exchange, Hsc70 returns to its ATP-bound form, and the substrate is subsequently released. Accessory proteins modulate different steps in the reaction cycle. DnaJ-like protein modulates the binding of substrates as well as stimulates ATP hydrolysis, while Hip stabilizes the Hsc70-ADP-substrate complex. Since ATP/ADP exchange is slow and no GrpE homologs have been found in the cytoplasm, some substrates would be released very slowly without help from accessory proteins. However, in the presence of p16, substrate can also be released from Hsc70-ADP, and the subsequent ATP/ADP exchange then converts Hsc70 to the form most accessible for substrate binding.

p16 also monomerizes Hsc70 oligomers and may counter the actions of Ydj1 on Hsc70. See “Discussion” for further details.

to the promoter region of c-myc oncogene (81), and inhibition of myeloid leukemic cell differentiation (82). In human tumors, reductions in Nm23 gene expression, another NDP kinase family member, have been associated with increased metastatic potential of a variety of carcinomas (reviewed in Ref. 83). However, none of these functions involve their NDP kinase activity. Recently, NDP kinase has been shown to function as a protein kinase that phosphorylates a serine residue on histone 2b or casein (84), and a histidine residue on ATP-citrate lyase (85). However, we did not observe any protein phosphorylation of Hsc70 by p16 in vitro (see “Results”). Therefore, the effect of p16 on Hsc70 is most likely based upon a protein-protein interaction resulting in a conformational change in both Hsc70 and p16.

NDP kinase is generally considered to be one of the “housekeeping” enzymes necessary for maintaining the nucleotide pools of the cell. One of the remarkable features of this enzyme seems to be its association with proteins that require guanine nucleotides for their functions. These proteins include initiation factor eIF2 (86), and GTP binding (G) proteins (87, 88). The ability of NDP kinase to convert GDP to GTP has led to the proposal that NDP kinase plays a role in the activation of G proteins in signal transduction by directly or indirectly providing GTP (89–91). Interestingly, plant NDP kinases have been proposed as a part of the signal transduction pathway during stress (92). NDP kinase from cultured sugarcane cells exhibits heat shock-stimulated autophosphorylation activity (93), and in tomato plant, its mRNA level is up-regulated in response to wounding (94). Since one of the most common signal transduction events during heat shock is protein phosphorylation, the up-regulated NDP kinase activity and protein levels may allow the plant to perceive and transduce signals related to high temperature stress (92). Since the protein levels of p16 increased when PLHC-1 cells were exposed to cadmium ions, it is possible that p16, like the plant NDP kinases, may also play a role in signal transduction pathways during stress. Furthermore, the direct association between p16 and Hsc70, combined with similar dose-dependent increases in both p16 and Hsc70/Hsp70 levels in response to cadmium ions reported here, demonstrate for the first time a direct link between NDP kinase and the stress response in animal cells.

The relationship between NDP kinase and the stress response is further demonstrated by the identification of NDP kinase as a plant flavonoid cromoglycate binding protein (95).
