Degradation of a protein via the ubiquitin system involves two discrete steps, signaling by covalent conjugation of multiple moieties of ubiquitin and degradation of the targeted substrate. Conjugation is catalyzed via a three-step mechanism that involves three distinct enzymes that act successively: E1, E2, and E3. The first two enzymes catalyze activation of ubiquitin and transfer of the activated moiety to E3, respectively. E3, to which the substrate is specifically bound, catalyzes formation of a polyubiquitin chain that is anchored to the targeted protein. The polyubiquitin-tagged protein is degraded by the 26 S proteasome, and free and reutilizable ubiquitin is released. In addition to the three conjugating enzymes, targeting of certain proteins requires association with ancillary proteins and/or post-translational modification(s). Using a specific antibody to deplete cell extract from the molecular chaperone Hsc70, we demonstrate that this protein is required for the degradation of actin, α-crystallin, glyceraldehyde-3-phosphate dehydrogenase, α-lactalbumin, and histone H2A. In contrast, the degradation of bovine serum albumin, lysozyme, and oxidized RNase A is Hsc70-independent. Mechanistic analysis revealed that the chaperone is required for the conjugation reaction; however, it does not substitute for E3. Involvement of the chaperone in the proteolytic process requires complex formation with the substrate. Formation of this complex appears to be essential in the proteolytic process. In addition, the proper function of the chaperone in the proteolytic process requires the presence of K⁺, which allows rapid cycles of dissociation and association of the complex. The chaperone may act by binding to the substrate and unfolding it to expose a ubiquitin ligase-binding site. In addition, it can also act directly on the ubiquitination machinery.

Degradation of short-lived and key regulatory proteins via the ubiquitin-proteasome pathway plays important roles in basic cellular processes. Protein targets of the ubiquitin system include, among others, cyclins, cyclin-dependent kinases and their inhibitors, tumor suppressors, oncoproteins, and transcriptional activators and their inhibitors. Selection of proteins for degradation can be mediated via primary (constitutive) or secondary signals such as post-translational modifications or via association with ancillary proteins. These signals are recognized by specific ubiquitin-protein ligases (E3), to which the substrate proteins bind prior to ubiquitination. Thus, the ligases play a key role in the ubiquitin proteolytic cascade, recognition and selection of proteins for conjugation and subsequent degradation. Following formation of the polyubiquitin adduct, the protein moiety is degraded by the 26 S proteasome complex, and free and reutilizable ubiquitin is released (reviewed in Refs. 1–4).

Molecular chaperones comprise a set of universally conserved proteins that bind and stabilize conformers of other proteins. A regulated, ATP-dependent association-dissociation cycle of the complex ensures the correct fate of the protein in vivo, which may be proper folding after synthesis, assembly into a multimeric complex, or translocation across a variety of intracellular membranes (5–8). Some of the chaperones are induced by stress, such as heat (heat shock proteins), and are probably involved in refolding of stress-induced denatured proteins. However, most chaperones are synthesized constitutively (heat shock cognate proteins) and play important roles in normal cellular processes.

Members of the 70-kDa family of molecular chaperones recognize hydrophobic domains that are exposed in non-native conformations; they do not bind to native proteins (9–11). To allow the multistep refolding process, the chaperone-substrate complex must undergo several ATP-dependent association-dissociation cycles. The substrate binds with high affinity to the heat shock cognate protein-ADP complex. Exchange of ADP with ATP lowers the affinity and results in the release of the substrate. The ATPase activity in the chaperone hydrolyzes the Hsc70-bound ATP to ADP, which results in rebinding of the released substrate (12). The dissociation of the substrate is cation-dependent (12). K⁺, for example, causes the conformational change in Hsc70 that is necessary for substrate dissociation; in the absence of the cation, MgATP favors the stable
complex conformation. Activation by cations correlates with the ionic radii of the ions: those that are within ~0.1 Å of that of K+, such as NH4+ and Rb+, are active. In contrast, those that are at least 0.3 Å smaller (Na+ and Li+) or larger (Cs+) are inactive (13).

It has been suggested recently that molecular chaperones may also be involved in intracellular protein degradation (reviewed in Ref. 14). In *Escherichia coli*, the non-secreted mutant form of alkaline phosphatase, phoA61, becomes associated with DnaK and is rapidly degraded by protease La (15). Proteolysis also requires DnaJ and GrpE, which have been shown to be involved in transport of the wild-type enzyme across the cell membrane. It was suggested that if the chaperones fail to successfully promote transport of the protein, they facilitate its rapid degradation. In another study, it has been shown that rapid degradation of abnormal proteins in *E. coli* involves GroEL and GroES (16). It is interesting to note that in prokaryotes, the general rate of protein degradation increases significantly following exposure to a heat stress. This increased proteolysis cannot be attributed only to stress-induced denaturation of intracellular proteins and provision of substrates to the proteolytic system(s). Rather, many of the bacterial proteases, such as the *lon* gene product, La (17), and the components of the Clp protease complex, ClpP, ClpA, ClpB, and ClpX (18, 19), are stress proteins that are induced concomitantly with generation of their respective substrates. In eukaryotes, molecular chaperones are involved in the degradation of proteins that is mediated by different pathways. In mitochondria, the chaperones Mdh1p and mt-Hsp70 cooperate with Pim1, the mammalian homolog of La and Clp, in the degradation of abnormal proteins (20). Selective and stress-induced degradation of proteins in lysosomes involves recognition by Hac73 of a peptide motif related to KFERQ. Researchers suggested that a complex between the chaperone and the recognition motif binds specifically to the lysosomal transmembrane glycoprotein LGP96, which serves, most probably, as a specific translocation channel (14, 21, 22). An intralysosomal chaperone, most likely also Hsc73, is required for the import of the substrate proteins into the lysosomal lumen (23). Stress proteins may be also involved in the removal of unassembled subunits of multimeric membrane complexes or extracellular proteins that are retained, due to a mutation, for example, in the secretory pathway. Researchers have shown that BiP, the Hsp70 homolog in the endoplasmic reticulum, is involved in the removal of a non-secreted variant of the immunoglobulin light chain (24).

Stress proteins have also been implicated in the degradation of proteins via the ubiquitin-proteasome pathway. A *Saccharomyces cerevisiae* cell that harbors a temperature-sensitive mutant of Ydj1 demonstrated a large defect in the overall breakdown of short-lived and abnormal proteins that is mediated by the ubiquitin system. Degradation of long-lived proteins that is mediated by the vacuole and proceeds in a ubiquitin-independent manner was unaffected (25). Interestingly, the effect was specific. Whereas the degradation of certain proteins such as several model “N-end rule” substrates and the mitotic cyclin Cbl5 was not affected, the degradation of other substrates (the transcriptional activator Gen4, for example) required Ydj1. Concomitantly, the ubiquitination of the Ydj1-dependent substrates was significantly reduced. Researchers also found that the chaperone generates a complex with its target substrate, but due to the limitations of the *in vitro* studies, they could not demonstrate that the complex serves as an essential intermediate in the proteolytic process (25). Degradation of the yeast cyclin Clb5 is also stimulated by Ydj1. However, in this case, the chaperone appears to stimulate phosphorylation of the cyclin by p34cdc28, a post-translational modification that signals the protein for conjugation and subsequent degradation by the ubiquitin system (26). Expression of several of the genes encoding components of the ubiquitin system is induced by heat. These include polyubiquitin genes in chick embryo fibroblasts (27) and yeast (28) and two of the yeast E2-encoding genes, UBC4 and UBC5 (29). Mutations in the UBC genes lead to defects that are characteristic of mutations in heat shock proteins, including temperature sensitivity and constitutive thermostolerance. Craig et al. (7) found that overexpression of UBP3, a ubiquitin C-terminal hydrolase, suppresses the ssa1saa2 phenotype. It is possible that lack of the chaperones leads to accumulation of misfolded proteins in the cytosol, and overexpression of a rate-limiting enzyme in the ubiquitin pathway ameliorates the situation by facilitating removal of these proteins. Ohba (30) demonstrated that a 70-kDa heat shock protein suppresses several of the defects caused by a mutation in the *S. cerevisiae* proteasome.

In this study, we show that Hsc70 is required for ubiquitin conjugation and subsequent degradation of certain proteolytic substrates *in vitro*. The chaperone is required in the conjugation step, and a complex between Hsc70 and the protein substrate serves, most probably, as an essential intermediate in the proteolytic process.

**EXPERIMENTAL PROCEDURES**

**Materials**

FPLC columns and Sepharose-immobilized protein G were from Pharmacia Biotech Inc. Materials for SDS-PAGE and Coomassie Blue stain and silver staining as well as molecular mass markers were from Bio-Rad. Histone H2A, hexokinase, and ATP-S were from Boehringer Mannheim. Lysozyme, BSA, GAPDH, α-crystallin, α-lactalbumin, ubiquitin, ovalbumin, phenylmethylsulfonyl fluoride, DTT, ATP, ATP-agarose, phosphocreatine, creatine kinase, 2-deoxyglucose, 2,6,10,14-tetra-methylpentadecane (pristane), immunoreactive rat IgG, Tris buffer, and HEPES were purchased from Sigma. Actin was from Worthington. DEAE-cellulose (DE52) was from Whatman. Ammonium sulfate was obtained from Life Technologies, Inc. Na125I was from DuPont NEN. Dulbecco’s modified Eagle’s medium and fetal calf serum were from Biological Industries (Kibbutz Ha’emek, Israel). All other chemicals were of high analytical grade.

**Methods**

Purification of Hsc70—Hsc70 was purified to almost homogeneity (>95%) from frozen bovine brain using Q-Sepharose Fast Flow FPLC anion-exchange chromatography, ATP-agarose affinity chromatography, and Mono Q FPLC anion-exchange chromatography essentially as described (9, 10). Briefly, a frozen brain (~500 g) was washed and homogenized in a blender with 1.2 volumes of buffer containing 10 mM HEPES-KOH, pH 6.5, 150 mM NaCl, 0.5 mM MgCl2, 1 mM EDTA, 0.5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Following low and high speed centrifugations to remove particulate material, the supernatant was dialyzed against Buffer A (25 mM Tris-HCl, pH 7.2, 0.1 mM EDTA, and 0.5 mM DTT) and loaded onto a Q-Sepharose Fast Flow column equilibrated in Buffer A. The adsorbed proteins were eluted in an FPLC apparatus (Pharmacia) using a linear gradient of 0–0.6 M KCl in Buffer A. Fractions containing Hsc70 as determined by SDS-PAGE and silver staining were pooled and dialyzed against Buffer B (20 mM HEPES-KOH, pH 7.0, 25 mM KC1, 10 mM Na2SO4, 0.1 mM EDTA, 0.5 mM DTT, and 2 mM MgCl2). The sample was applied to an ATP-agarose column equilibrated in Buffer B, and Hsc70 was eluted by washing the column with Buffer B containing 5 mM MgATP. Fractions containing Hsc70 (see above) were pooled and equilibrated by dialysis in Buffer A. The pooled fractions were applied to a Mono Q anion-exchange chromatography column equilibrated in Buffer A. Hsc70 was eluted by a linear gradient of 0.1–0.6 M KCl in Buffer A, and fractions containing the chaperone were pooled, dialyzed against Buffer B lacking MgCl2 and stored in small aliquots at ~70 °C. All purification procedures were carried out at 2–4 °C.

Monoclonal Anti-Hsc70 Antibodies—Rat monoclonal anti-Hsc70 antibodies were prepared from IB5 rat hybridoma cells (31). Cells were grown as a monolayer at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum. Cultures were harvested, and the cells were washed twice with phosphate-buffered saline. Washed
cells were injected (5 × 10^6 cells in 2 ml) into male Harlan Sprague Dawley rats that had been injected intraperitoneally with preteinase 8 days earlier. Ascites fluid, developed within 1 week, was collected and subjected to precipitation by 45% (NH₄)₂SO₄ followed by dialysis against a buffer containing 25 mM Tris-HCl, pH 7.2, and 75 mM NaCl. Immunodepletion of Hsc70—300-μl aliquots of reticulocyte lysate (see below; contains ∼30 μg of Hsc70 as determined by quantitative Western blot analysis) were incubated at 4 °C for 90 min in the presence of 125 μg of IB5 antibody (prepared as described above) or 125 μg of preimmune rat IgG. Following incubation, the mixtures were added to 25 μl of packed protein G-Sepharose beads (washed with 20 mM Tris-HCl, pH 7.2, and 75 mM NaCl) and swirled at 4 °C for 45 min. After a brief centrifugation to remove the beads, the supernatants were tested for the presence of Hsc70 by Western blot analysis. Detection was performed with the ECL system (Amersham Corp.) using IB5 as the primary antibody and rabbit anti-rat peroxidase-conjugated IgG as the secondary antibody. Similarly, Hsc70 was depleted also from 100-μl aliquots of Fraction I or IIA (see below; both also contain ∼30 μg of Hsc70).

**Preparation of Substrates—**RCRM-α-LA was prepared as described (32). 125I-labeled proteins were prepared by the chloramine-T method as described (32).

Preparation of Rabbit Reticulocyte Lysate and Conjugating Enzymes—Reticulocyte-rich blood was induced in rabbits by successive injections of phenylhydrazine, and reticulocyte lysate was prepared as described (33). Lysates were resolved by anion-exchange chromatography on DEAE-cellulose into unadsorbed material (Fraction I) and high salt eluate (Fraction II) as described (33). E1 was purified to homogeneity from Fraction II following affinity chromatography on immobilized ubiquitin as described (33). E2-F1 was purified to homogeneity from Fraction II following affinity chromatography on immobilized ubiquitin as described (33). E2-F1 (or UbcH5), and the indicated amount of Fraction IIA protein.

**Formation of the Hsc70-Protein Substrate Complex—**For monitoring complex formation between the chaperone and the substrates, Hsc70 and the various 125I-labeled protein substrates (0.1 μg, −10^4 cpm) were added. To provide energy, conjugation assays contained 5 mM ATP, whereas degradation assays contained ATP/KCl regenerating system. Complete lysate + ATP represents a system that was preincubated for 5 min at 37 °C in the presence of 2-deoxyglucose and hexokinase prior to addition of the labeled substrate.

**Determination of Protein—**Protein concentration was determined by the Bradford method (38) using BSA as a standard.

**RESULTS**

**Effects of Cations on the Degradation of Certain Protein Substrates of the Ubiquitin System**—We noted that the degradation in reticulocyte lysate of 125I-labeled actin, α-crystallin, histone H2A, GAPDH, α-LA, and RCM-α-LA is inhibited following a short period of dialysis in low molecular mass cation dialysis tubing (molecular mass cutoff = 1.0 kDa). Addition of KCl reconstituted the inhibited activity (Table I). The degradation of BSA, lysozyme, and oxidized RNase A was not affected by the removal and resupplementation of KCl (Table I; results for oxidized RNase A are not shown). Titration of the cation shows that the maximal stimulating effect is attained at −20 mM (Fig. 1; see “Discussion”). We have previously shown that all these substrates are targeted for degradation by the

| Substrate | Complete lysate + ATP | −ATP | Dialyzed lysate + ATP | +ATP/KCl |
|-----------|-----------------------|------|-----------------------|----------|
| Actin     | 25.1                  | 4.7  | 7.2                   | 26.5     |
| α-Crystallin | 16.4                | 5.9  | 4.1                   | 15.6     |
| Histone H2A | 29.6                | 3.8  | 2.6                   | 11.6     |
| GAPDH     | 24.5                  | 2.6  | 9.1                   | 36.8     |
| α-LA      | 17.3                  | 7.3  | 3.4                   | 15.6     |
| RCM-α-LA  | 39.1                  | 9.1  | 2.6                   | 41.6     |
| BSA       | 43.0                  | 10.9 | 4.1                   | 27.3     |
| Lysozyme  | 31.8                  | 27.3 | 2.6                   | 27.8     |

**TABLE I**

**Effect of K** on the degradation of protein substrates of the ubiquitin proteolytic system

Degradation of the various 125I-labeled substrates was monitored by the release of radioactivity into trichloroacetic acid-soluble fractions as described under “Experimental Procedures.” Complete lysate + ATP represents a system that was incubated in the presence of ATP and an ATP-regenerating system. Complete lysate − ATP represents a system that was preincubated for 5 min at 37 °C in the presence of 2-deoxyglucose and hexokinase prior to addition of the labeled substrate.

| Substrates of the Ubiquitin System— |  |
|------------------------------------|--|
| Actin                              |  |
| α-Crystallin                       |  |
| Histone H2A                        |  |
| GAPDH                             |  |
| α-LA                              |  |
| RCM-α-LA                          |  |
| BSA                                |  |
| Lysozyme                          |  |

**Substrates of the Ubiquitin System—**

- Actin
- α-Crystallin
- Histone H2A
- GAPDH
- α-LA
- RCM-α-LA
- BSA
- Lysozyme

**Substrates of the Ubiquitin System—**

- Actin
- α-Crystallin
- Histone H2A
- GAPDH
- α-LA
- RCM-α-LA
- BSA
- Lysozyme

**Substrates of the Ubiquitin System—**

- Actin
- α-Crystallin
- Histone H2A
- GAPDH
- α-LA
- RCM-α-LA
- BSA
- Lysozyme
ubiquitin proteolytic system in vitro (32, 34, 39, 40). In an attempt to characterize better the cations that can reconstitute proteolysis, we found that NH$_4^+$ can substitute for K$^+$ efficiently, Rb$^+$ is less effective, Cs$^+$ is significantly less active, and Na$^+$ and Li$^+$ are inactive (Fig. 1 and Table II). In a search for a potential role of the cations in the proteolytic process, we noted that several functions of the 70-kDa heat shock cognate protein (Hsc70), such as peptide binding and release and clathrin uncoating, require K$^+$ (12, 13, 41). The cation is required, most probably, to allow the change in the conformation of the chaperone that is necessary for substrate dissociation. Examination of the effect of different monovalent cations on the activity of Hsc70 revealed an identical profile to that observed for stimulation of ubiquitin-dependent degradation (Ref. 13 and Table II). Thus, we set out to examine directly the effect of Hsc70 on the function of the ubiquitin system.

**Effect of Hsc70 on Selective Degradation of Protein Substrates by the Ubiquitin Cell-free System**—To test the hypothesis that the cation requirement for proteolysis reflects a requirement for the molecular chaperone Hsc70, we used a monoclonal antibody to deplete Hsc70 from reticulocyte lysate, the proteolytic extract that is used most frequently for monitoring degradation in a cell-free system. As can be seen in Fig. 2, treatment of the extract with the specific antibody results in almost complete removal of Hsc70. Quantitative assessment of the chaperone in the lysate revealed a concentration of $\sim$100 $\mu$g/ml. Depletion of Hsc70 resulted in significant inhibition of

![Fig. 1. Cation-dependent degradation of actin and BSA. Degradation of $^{125}$I-labeled actin (○, ■) and BSA (■) was monitored in a dialyzed reticulocyte lysate in the presence of increasing concentrations of K$^+$ (○, ■), NH$_4^+$ (○), or Na$^+$ (■). Degradation was monitored by measuring the release of radioactivity into trichloroacetic acid-soluble fractions as described under "Experimental Procedures." 100% reflects the release of radioactivity into the supernatant. As can be seen in Figs. 3A and 4A, removal of ions inhibits conjugation of GAPDH and α-crystallin, respectively. Addition of K$^+$ reconstitutes adduct formation. Similarly, addition of NH$_4^+$ and to a lesser extent, Rb$^+$ also restores activity (data not shown). As demonstrated in Figs. 3B and 4B, depletion of Hsc70 also inhibits conjugate formation. Resupplementation of the chaperone restores activity. Fig. 5 demonstrates that the conjugation of actin requires both K$^+$ and Hsc70;**

| Substrate | Complete lysate | Dialyzed lysate | Dialyzed lysate |
|-----------|----------------|----------------|----------------|
|           |                | Complete lysate| + K$^+$ | + NH$_4^+$ | + Rb$^+$ | + Cs$^+$ | + Na$^+$ | + Li$^+$ |
| Actin     | 22.2           | 3.7            | 23.8      | 24.5      | 15.6     | 5.2     | 3.1     | 2.9     |
| α-Crystallin | 15.7          | 1.9            | 16.7      | 13.2      | 11.5     | 4.7     | 2.2     | 1.4     |
| Histone H2A | 21.6          | 3.2            | 24.7      | 18.9      | 13.2     | 5.6     | 3.0     | 2.1     |
| GAPDH    | 19.5           | 3.3            | 18.4      | 16.7      | 12.9     | 6.9     | 4.1     | 2.8     |
| RCM-α-LA | 34.7           | 6.4            | 30.1      | 28.6      | 17.6     | 10.4    | 7.6     | 6.9     |
addition of any of these components alone is not sufficient to promote conjugation. Similar to the effect of ions and Hsc70 on degradation (Fig. 1 and Tables I and III), the effects of these factors on conjugation are also specific for a certain subset of substrates: dialysis and depletion of Hsc70 do not affect conjugation of lysozyme (Fig. 6) and oxidized RNase A (data not shown). The conjugating system contained either ATPγS or ATP (see “Experimental Procedures”). The ATP analog is utilized in conjugation assays since it promotes conjugation (via activation of E1), but inhibits degradation (since the 26 S proteasome can utilize only ATP), and therefore leads to accumulation of ubiquitin adducts (42). We were surprised to find that the nonhydrolyzable nucleotide analog is active, although not more than ATP, in promoting Hsc70- and cation-dependent conjugation that is apparently dependent on ATP hydrolysis. This may be due to the presence of endogenous ATP and ADP in the lysate and to the activity of adenylate kinase that generates ATP from ADP. Slight hydrolysis of ATPγS can also generate ADP. Also, the vast molar excess of the chaperone over the labeled substrate may allow presentation of the substrate to the conjugating machinery after only a single cycle of association. Thus, the reaction may not be heavily dependent upon the presence of ATP. We noted that addition of Hsc70 to a complete lysate stimulated conjugation to α-crystallin (Fig. 4B, compare lanes 3 and 4) and actin (data not shown). In contrast, addition of the chaperone had no effect on the conjugation of GAPDH (Fig. 3B, compare lanes 3 and 4), histone H2A, α-LA, and RCM-α-LA (data not shown). As noted before, resupplementation of excess Hsc70 had a stimulatory effect on the degradation of these substrates (Table III). These results can be explained by the possibility that the affinity between actin and crystallin and the chaperone is weaker than that of the other protein substrates. Most of the endogenous chaperone contained in the lysate is occupied by native cellular proteins. Therefore, exogenous substrates with low affinity cannot displace the endogenous substrates from the chaperone and are partially dependent for their conjugation and degradation upon the addition of excess exogenous and substrate-free Hsc70. The high affinity substrates can displace the endogenous proteins and are not dependent upon the addition of free exogenous chaperone for their conjugation and subsequent degradation.

**Conjugation Requires, in Addition to Hsc70, All Three Conjugating Enzymes, E1, E2, and E3**—Conjugation of proteolytic substrates involves specific complex formation with their cognate E3 enzymes, which serve, among other functions, as docking proteins for the substrates during the tagging process. Thus, it was of interest to study whether the molecular chap-

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**TABLE III**

**Effect of depletion and resupplementation of Hsc70 on the degradation of actin, α-crystallin, GAPDH, RCM-α-LA, and lysozyme**

| Substrate | Complete lysate | Hsc70-depleted lysate | Hsc70-depleted lysate + Hsc70 |
|-----------|----------------|-----------------------|-----------------------------|
| Actin     | 20.8           | 3.7                   | 25.2                        |
| α-Crystallin | 12.6         | 2.3                   | 18.0                        |
| GAPDH     | 22.7           | 6.2                   | 18.5                        |
| α-LA      | 16.2           | 10.2                  | 14.5                        |
| RCM-α-LA  | 42.7           | 8.1                   | 37.3                        |
| Lysozyme  | 26.8           | 25.2                  | 24.9                        |

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**TABLE IV**

**Effect of depletion and resupplementation of K+ and Hsc70 on the degradation of actin**

| Substrate | Complete lysate | Dialyzed and Hsc70-depleted lysate | Dialyzed and Hsc70-depleted lysate + K+ | +Hsc70 | +K+/Hsc70 |
|-----------|----------------|-----------------------------------|----------------------------------------|--------|----------|
| Actin     | 22.7           | 4.9                               | 7.1                                    | 5.3    | 26.2     |

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**Fig. 2. Immunodepletion of Hsc70 from reticulocyte lysate.** Hsc70 was removed from rabbit reticulocyte lysate via immunodepletion, and Hsc70 in the extract was detected following SDS-PAGE, Western blot analysis, and ECL as described under “Experimental Procedures.” Lanes 1 and 2, 1 and 0.5 μl of untreated lysate resolved on the gel, respectively; lanes 3 and 4, same as lanes 1 and 2, except that the lysate was treated with control rat IgG; lanes 5 and 6, same as lanes 1 and 2, except that the lysate was treated with anti-Hsc70 antibody 1B5. 97.4 and 69.0 denote migration of the molecular mass markers phosphatase b and BSA, respectively (in kilodaltons). The site of migration of Hsc70 is marked.

**Fig. 3. Effect of K+ (A) and Hsc70 (B) on the conjugation of ubiquitin to 125I-GAPDH.** A, conjugation was monitored as described under “Experimental Procedures” in a complete lysate without ATP (lane 2), a complete lysate containing ATPγS (lane 3), a dialyzed lysate containing ATPγS and 10 mM KCl (lane 5). Lane 1 represents the labeled, untreated substrate. B, conjugation was monitored as described under “Experimental Procedures” in a complete lysate without ATP (lane 2); a complete lysate with ATPγS (lane 3); a complete lysate containing ATPγS and 3.0 μg of purified Hsc70 (lane 4); an Hsc70-immunodepleted lysate containing ATPγS (lane 5); and a similar lysate to which purified Hsc70 was added at 0.3 (lane 6), 1.0 (lane 7), and 3.0 (lane 8) μg. Lane 1 represents the labeled, untreated substrate. 125I-GAPDH denotes the labeled substrate. Conj., conjugates; Ori., origin of gel; D.F., dye front. Molecular mass markers are myosin (200.0 kDa), phosphorylase b (97.4 kDa), BSA (69.0 kDa), ovalbumin (46.0 kDa), and carbonic anhydrase (30.0 kDa).
erone, which also recognizes specific motifs in the substrate, can substitute for E3 in the conjugation reaction. In this case, catalysis of conjugation would require E1, E2, and the chaperone, but not E3. As demonstrated in Fig. 7 (lanes 1 and 2), addition of E1, E2-F1 (or UbcH5; data not shown), and the chaperone to a cell-free reconstituted system is not sufficient to restore conjugation. Conjugate formation is dependent upon the addition of Fraction IIA (lanes 3 and 4), which contains all the known E3 enzymes (34, 36, 43). Depletion of Hsc70 from Fraction IIA inhibits conjugation (lane 5), whereas, as expected, addition of purified Hsc70 to the system restores activity. Thus, Hsc70 cannot function as an E3 enzyme and is not involved directly in the conjugation process.

Possible Mechanism of Involvement of Hsc70 in Ubiquitin-mediated Proteolysis: Formation of a Chaperone-Substrate Intermediate Complex—All known activities of molecular chaperones are mediated via complex formation with their substrates. Thus, it was important to determine whether Hsc70 generates a complex with the chaperone-dependent proteolytic substrates, but not with those that are degraded in a chaperone-independent manner. As can be seen in Fig. 8, α-LA and RCM-α-LA, two proteins that require Hsc70 for their conjugation and subsequent degradation (see Table III; results for conjugation of both proteins are not shown), generate a complex with Hsc70. Similarly, actin, α-crystallin, GAPDH, and histone H2A associate with Hsc70, whereas BSA, oxidized RNase A, and lysozyme do not (data not shown; association of Hsc70 with these proteins was followed by monitoring the shift of radioactivity of these proteins to a higher molecular mass region of a gel filtration chromatography column as described under “Experimental Procedures”). It is interesting to note that a larger proportion of RCM-α-LA became associated with the chaperone compared with the nonmodified protein (Fig. 8, compare lanes 2 and 4). This difference is probably due to the higher affinity that the completely unfolded protein has for the chaperone. This high affinity is most probably reflected also in the greater sensitivity of the modified protein to cation (Table I) and Hsc70 (Table III) depletion. Hsc70-protein complexes were also obtained when the substrates were incubated in the presence of a complete proteolytic extract (devoid of ATP to prevent conjugation and degradation) (data not shown). Utilization of Hsc70-depleted lysate did not yield a high molecular mass complex (data not shown). This observation demonstrates that the only protein in the mixture that associates with the substrate is the chaperone.
We have shown that the conjugation and degradation of certain proteolytic substrates by the ubiquitin system in vitro require $K^+$ and the molecular chaperone Hsc70. Immunodepletion of the protein from reticulocyte lysate results in a significant inhibition of conjugation and subsequent proteolysis of actin, a-crystallin, GAPDH, histone H2A, a-LA, and RCM-a-LA. In contrast, the conjugation and degradation of yet another set of proteolytic substrates (BSA, lysozyme, and oxidized RNase A) are chaperone-independent. The chaperone forms a complex with the target substrate that serves as an essential intermediate in the proteolytic process. However, this complex cannot substitute for E3, which also forms a complex with the substrate prior to tagging it with ubiquitin. We noted that the chaperone is required for formation of high molecular mass ubiquitin conjugates of histone H2A and RCM-a-LA; formation of low molecular adducts of these proteins is Hsc70-independent (data not shown). It is interesting to note that formation of low molecular mass conjugates is also E3-independent and requires only E1 and E2 (reviewed in Ref. 4). Also, only the high molecular mass adducts serve as proteolytic substrates for the 26 S proteasome complex. Taken together, these findings indicate that the chaperone and E3 act in concert to generate the appropriate multiply ubiquitinated substrates that are recognized and subsequently proteolyzed by the 26 S proteasome (see also below). While the cations are most probably required for the activity of the chaperone, it is still possible that the two components act via two distinct and independent mechanisms. However, as noted, the identity between the cation profiles required for Hsc70 activation and proteolysis makes this possibility unlikely.

The mechanistic basis for the substrate specificity of Hsc70 is not known. It may be due to specific structural features of the proteins that allow their binding to the chaperone. We noted that all three “Hsc70-independent” substrates (lysozyme, BSA,
and oxidized RNase A) are primary “N-end rule” proteins that are recognized and targeted via direct binding of their free N-terminal and “destabilizing” amino acid residue (44). It is possible that in these proteins, the recognition domain that is extremely short and well defined is exposed and is directly recognized by the ubiquitin-protein ligase E3. Therefore, chaperone-assisted unfolding of the protein to expose the E3 recognition domain is not necessary. Structural analysis of labeled lysozyme has shown that at least for this protein, the N-terminal domain is indeed freely accessible (45). For the Hsc70-dependent substrates, it is possible that their E3 recognition site is hidden, and the binding to the chaperone is necessary to uncover it. Thus, for certain substrates, the rate-limiting step in the multistep ubiquitin cascade may be their recognition by the ligase. The rate of this step may vary from substrate to substrate, and the role of the chaperone is to facilitate recognition for the “chaperone-dependent” substrates.

For other substrates, this may not be the rate-limiting step, and their recognition proceeds unimpaired even in the absence of the chaperone and the ion. An alternative explanation for the selective effect of Hsc70 may involve the degree of unfolding (denaturation) or the aggregation state of the substrate. It is clear that, at least for the pair α-LA and RCM-α-LA, there is a strong and direct correlation between the degree of unfolding of the protein and its sensitivity to cation and Hsc70 depletion: completely unfolded RCM-α-LA shows a greater dependence on K+ and Hsc70 for its conjugation (data not shown) and subsequent degradation compared with its cognate, untreated protein (Tables I and III). It also associates at a higher efficiency with the chaperone (Fig. 8, compare lanes 2 and 4). In a different approach to the problem of “substrate specificity,” we noted that the chaperone-dependent (but not the chaperone-independent) substrates appear to be in an aggregated form (as determined by the proportion of the substrate that can be precipitated by a short, high speed centrifugation). Our initial experiments indicate that Hsc70 may be involved in solubilization of these aggregates (data not shown). It should be noted that in the cell, Hsc70 may serve to prevent aggregation rather than to resolubilize aggregated proteins. It is clear that all the substrates we utilized are denatured to a smaller or larger degree, but the association of the chaperones with proteins appears to be mostly with unfolded proteins. Prydman and Hartl (46) noted recently in their study on the association of chaperones with nascent truncated actin that although caution should be exercised when extrapolating from in vitro studies to the functions of chaperones in the cell, the interactions of chemically denatured proteins with chaperones may resemble those occurring in the cell following its exposure to various forms of stress such as elevated temperature.

Our findings also indicate that the involvement of the chaperone in the proteolytic process requires complex formation with the targeted substrate and that the complex that serves as an essential intermediate in the proteolytic process must undergo cation-dependent cycles of association-dissociation. Like other proteins that bind to Hsc70, it is possible that without the cation, the proteolytic substrate is “locked” into the chaperone and therefore cannot be released for presentation to the conjugating machinery.

An important problem involves the mechanism of action of the chaperone in the proteolytic system. One possibility is that Hsc70 binds the substrate and actively unfolds it in a manner that exposes the ligase-binding domain. The cation is required for release of the substrate from the chaperone and its transfer to the ligase. Here, the chaperone serves to “hold” the substrate in an unfolded, but E3-bindable state. An alternative (and apparently antithetical) explanation involves repeated cycles of binding and release with an attempt to refold the protein to its native form. It is highly likely that many cellular proteins are denatured to a certain degree. One well known and carefully studied function of molecular chaperones involves refolding of denatured proteins to their native form. The process is gradual and involves repeated, cation-dependent cycles of association-dissociation (5, 12, 13). It is possible that Hsc70 is involved in an attempt to refold denatured proteolytic substrates. Failure to renature the proteins leads to their presentation to the ligase. The “decision” to present the denatured protein to the scavenging system may be based on the rate of successful collisions between the Hsc70-substrate complex and E3. A successful collision is one in which the chaperone presents to the ligase a protein with an exposed recognition domain and that results in transfer of the chaperone-bound substrate to E3. Successful refolding of the protein to a form in which this domain is no longer exposed will lead to an infertile collision and eventually to release of a native refolded protein from the chaperone. Failure to refold the protein and continuous exposure of the ligase recognition motif increase the chances for a successful collision. This “refold or degrade” hypothesis is clearly distinct from the first, “holding” hypothesis. Kinetic measurements as well as utilization of different chaperones and proteolytic substrates in the cell-free system can be now used to test these two mechanistic hypotheses. An additional explanation for the involvement of the chaperone in the proteolytic process is that it affects, in addition to the substrate, one or more of the components of the system. It should be noted that the identity of the E3 enzymes that are involved in the conjugation of all the chaperone-dependent proteins is not known. Therefore, experiments with purified components are not possible at this stage, and a detailed analysis of the mechanism of involvement of Hsc70 in ubiquitin-mediated proteolysis will have to await further characterization of the system.

It is not known whether Hsc70 affects only conjugation or whether it is involved also in degradation of the conjugates by the 26 S proteasome. Also, it is not known whether, as in protein synthesis (as determined in a cell-free reconstituted system and not in the intact cell), other chaperones are also involved in the proteolytic process. Folding of nascent polypeptide chains requires high molecular mass assembly with Hsc70, Hsc40, and TRiC (47). The three chaperones are required for cotranslational formation of all the domains of the protein and completion of folding following release of the chain from the ribosome. Depletion of any of the chaperones leads to improper folding of the nascent chain and release of an inactive protein.

### Table V

Degradation of 125I-RCM-α-LA and of Hsc70-bound 125I-RCM-α-LA in reticulocyte lysate

| Substrate                       | Complete lysate | Dialyzed and Hsc70-depleted lysate | Dialyzed and Hsc70-depleted lysate |
|--------------------------------|----------------|------------------------------------|------------------------------------|
|                                | ATP            | +ATP                               | +K-                                 |
| RCM-α-LA                       | 4.5            | 43.2                               | 7.4                                 |
| RCM70-RCM-α-LA complex         | 3.6            | 39.6                               | 5.9                                 |

Degradation of 125I-RCM-α-LA was generated following incubation of the labeled substrate and the chaperone and isolation of the complex via gel filtration chromatography as described under “Experimental Procedures.” Degradation of substrates was monitored as described in the legends to Tables I–IV and under “Experimental Procedures.”
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REFERENCES
1. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 65, 801–847
2. Hochstrasser, M. (1995) Curr. Opin. Cell Biol. 7, 215–223
3. Deshaies, R. J. (1995) Trends Cell Biol. 5, 428–434
4. Ciechanover, A. (1994) Cell 79, 13–21
5. Hendrick, J. P., and Hartl, F.-U. (1995) Annu. Rev. Genet. 29, 437–486
6. Craig, E. A., Baxter, B. K., Becker, J., Halladay, J., and Ziegelhoffer, T. (1994) Trends Cell Biol. 4, 13–21
7. Hendrick, J. P., and Hartl, F.-U. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 137–151
8. Sadis, S., and Hightower, L. E. (1992) Biochemistry 31, 9406–9412
9. Sadis, S., and Hightower, L. E. (1992) Biochemistry 31, 9406–9412
10. Sadis, S., and Hightower, L. E. (1992) Biochemistry 31, 9406–9412
11. Fourie, A. M., Sambrook, J. F., and Gething, M.-J. H. (1994) J. Biol. Chem. 269, 2025–2033
12. Palleros, D. R., Reid, K. L., Shi, L., Welch, W. J., and Fink, A. L. (1993) J. Biol. Chem. 268, 664–666
13. Forsythe, R. W., and Hightower, L. E. (1992) Biochemistry 31, 9406–9412
14. O’Brien, M. C., and McKay, D. B. (1995) J. Cell Biol. 131, 7106–7116
15. O’Brien, M. C., and McKay, D. B. (1995) J. Cell Biol. 131, 7106–7116
16. O’Brien, M. C., and McKay, D. B. (1995) J. Cell Biol. 131, 7106–7116
17. Craig, E. A., Baxter, B. K., Becker, J., Halladay, J., and Ziegelhoffer, T. (1994) Trends Cell Biol. 4, 13–21
18. Craig, E. A., Baxter, B. K., Becker, J., Halladay, J., and Ziegelhoffer, T. (1994) Trends Cell Biol. 4, 13–21
19. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
20. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
21. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
22. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
23. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
24. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
25. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
26. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
27. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
28. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
29. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
30. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
31. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
32. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
33. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
34. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
35. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
36. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
37. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
38. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
39. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
40. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
41. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
42. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
43. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
44. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
45. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
46. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
47. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
48. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
49. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
50. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
51. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
52. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
53. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
54. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
55. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
56. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
57. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
58. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
59. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
60. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
61. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
62. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
63. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
64. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
65. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
66. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
67. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
68. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
69. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
70. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
71. Ciechanover, A. (1994) J. Biol. Chem. 269, 2025–2033
Ubiquitin-dependent Degradation of Certain Protein Substrates in Vitro Requires the Molecular Chaperone Hsc70
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