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Mammalian Hsp70 and Hsp110 Proteins Bind to RNA Motifs Involved in mRNA Stability*

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In this study, *in vitro* RNA binding by members of the mammalian 70-kDa heat shock protein (Hsp) family was examined. We show that Hsp/Hsc70 and Hsp110 proteins preferentially bound AU-rich RNA *in vitro*. Inhibition of RNA binding by ATP suggested the involvement of the N-terminal ATP-binding domain. By using deletion mutants of Hsp110 protein, a diverged Hsp70 family member, RNA binding was localized to the N-terminal ATP-binding domain of the molecule. The C-terminal peptide-binding domain did not bind RNA, but its engagement by a peptide substrate abrogated RNA binding by the N terminus of the protein. Interestingly, removal of the C-terminal α-helical structure or the α-loop domain unique to Hsp110 immediately downstream of the peptide-binding domain, but not both, resulted in considerably increased RNA binding as compared with the wild type protein. Finally, a 70-kDa activity was immunoprecipitated from RNA-protein complexes formed *in vitro* between cytoplasmic proteins of human lymphocytes and AU-rich RNA. These findings support the idea that certain heat shock proteins may act as RNA-binding entities *in vivo* to guide the appropriate folding of RNA substrates for subsequent regulatory processes such as mRNA degradation and/or translation.

Mammalian cells respond to a variety of stress conditions with elevated synthesis of a remarkably similar set of proteins, called the stress or heat shock proteins (Hsp)[1] (1, 2). Among these, members of the ATP-binding 70-kDa Hsp family are one of the most highly conserved proteins known in biology with greater than 50% overall amino acid sequence homology between *Escherichia coli* and man (3, 4). A large body of evidence has emerged during the past decade which indicates that members of the 70-kDa Hsps may mediate crucial cellular functions under non-stress conditions, such as the clathrin uncoating in mammalian cells (5), post-translational translocation of proteins to the endoplasmic reticulum lumen (6), selective import of proteins into lysosomes (7), or folding assistance to nascent polypeptides during translation (8–10). As well, the *E. coli* 70-kDa Hsp homologue, DnaK, was demonstrated to mediate a DNA helicase function in λ phage replication in association with additional protein components (11).

A unique subfamily of mammalian Hsp70 proteins comprises large stress response proteins, members of the Hsp110/SSE subfamily. The mammalian Hsp110 protein has been cloned from Chinese hamster ovary cells and characterized in detail in our laboratory, and a number of unique features that distinguish this diverged member from the DnaK-Hsp70 family have been identified (12–13). One of the most prominent structural elements present in Hsp110 and related proteins is a 100-amino acid-long α-helical loop found between the peptide-binding domain and the C-terminal α-helical region (12). Hsp110 has been shown to protect heat-denatured proteins from aggregation as well as to confer cellular thermoresistance *in vivo* (13).

It is widely believed that the primary function of the four major groups of heat shock proteins (Hsp27, Hsp60, Hsp70, and Hsp90) is to assist protein folding *in vivo.* Functions include the initial folding of newly synthesized proteins to refolding of proteins damaged by environmental stress. A common theme in this molecular chaperoning function is the transient binding or association of Hsp proteins with partially folded or misfolded peptide stretches of proteins (14). In case of Hsp70, binding of ATP by the N-terminal domain facilitates association of the peptide substrate, whereas ATPase activity of the chaperone is required for substrate release (15).

Little is known, however, if molecular targets other than proteins exist that interact with members of the Hsp70 protein family and additional molecular chaperones. Several lines of evidence suggest that, in the cell, these proteins also recognize and bind macromolecules other than proteins. For example, it has recently been demonstrated that GroEL, the *E. coli* Hsp60 homologue, can associate with lipid membranes while remaining functional as a protein folding chaperone (16). This mechanism has been proposed to be a membrane-protective component during heat stress (16). Equally important, heat shock proteins have been recently associated with various aspects of RNA metabolism. GroEL has been suggested to be part of a protein complex that protects bacterial transcripts from RNase E-mediated degradation (17, 18). Intriguingly, Miczak and coworkers (19) demonstrated that DnaK and in some instances GroEL co-purify with the bacterial degradosome, although no functional implications have been proposed in mRNA metabolism. The 60-kDa chaperonin of the thermophilic archaeon,

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[1] The abbreviations used are: Hsp, heat shock protein; PIPES, 1,4-piperazinediethanesulfonic acid; UTR, untranslated region; ARE, AU-rich instability determinant element; IFN, interferon; IL, interleukin; hu, human; PAGE, polyacrylamide gel electrophoresis; RCMLA, reduced carboxymethylated lactalbumin.

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Sulfolobus solfataricus has been identified as an RNA-binding protein with specific interaction with the 16 S rRNA (20). Additionally, an *in vitro* reconstituted endonuclease activity is associated with this chaperonin, which cleaves pre-rRNA at a specific 5′ site (20). Earlier studies on heat response regulation in *Drosophila* demonstrated that expression of the major heat-induced protein, Hsp70, is self-regulated at both the transcriptional and post-transcriptional levels (21). Binding of Hsp70 to its own mRNA has been proposed to explain the rapid alterations in mRNA stability observed in the cytoplasm (21). Finally, a previously described 102-kDa RNA-binding protein, conserved throughout higher plants, has recently been identified as being identical with Hsp101 (22). Through direct binding and complex formation with the 5′ leader sequence of tobacco mosaic viral RNA, Hsp101 acts as a translational enhancer, a function that can be recapitulated in yeast and that is independent of its role in conferring thermoresistance (22). Each of these studies independently implicates the involvement of various heat shock proteins in regulatory mechanisms of RNA metabolism through their direct or indirect association with RNA.

Our previous label transfer studies using cytoplasmic lysates of human lymphocytes demonstrated that a 70-kDa complex was invariably detected with AU-rich 3′-UTR RNA sequences of various lymphokine and proto-oncogene mRNAs (see *e.g.* in Ref. 23). In addition, during attempts of expression and purification of AU-rich RNA-binding fusion proteins in *E. coli*, an intense 70-kDa activity was co-purified with these proteins. Based on these results, we tested AU-rich RNA binding capacity of mammalian Hsp/Hsc70 proteins. In this study we report direct RNA binding of mammalian Hsp70 and Hsp110 molecules by their N-terminal ATP-binding region. We demonstrate that these proteins preferentially bind AU-rich 3′-UTR sequences and that this interaction can be regulated by ATP at physiological concentrations. We also show that engagement of the peptide-binding domain by a peptide substrate abrogates RNA binding by the N terminus of the protein. Additionally, RNA binding activity and sequence specificity can be modified by deletion or rearrangement of C-terminal domain structures. Finally, Hsp70 proteins can be immunoprecipitated from protein-RNA complexes formed between cytoplasmic proteins of human lymphocytes and AU-rich RNA probes. These results suggest that binding of Hsp70 family members to 3′-UTR ARE sequences may be mechanistically important in the cytoplasmic metabolism of lymphokine and other short-lived mRNAs.

EXPERIMENTAL PROCEDURES

Reagents—RPMI and Dulbecco's modified Eagle's tissue culture media, protein A-Sepharose, bovine serum albumin, reduced carboxymethylated lactalbumin (RCMLA), and homoribopolymers (poly(A), poly(C), poly(G), and poly(U)) were purchased from Sigma. Fetal calf serum was from Protein GMK., Hungary. Recombinant Hsp proteins and monoclonal antibody against human Hsp70 were from StressGen Biotechnologies. [^{32}P]UTP (3000 Ci/mmol) was purchased from Izotóp Kft., Hungary, and from NEN Life Science Products. Unlabeled nucleotides were from Roche Molecular Biochemicals, Germany.

Generation of Recombinant Hsp110 Proteins—Hsp110, a diverged member of the Hsp70 stress protein family, was cloned and identified earlier in our laboratory (12). Both the wild type and targeted deletion mutants of Hsp110 were expressed in *E. coli* as His-tagged proteins as described elsewhere (24).

Cell Culture and Cytoplasmic Lysate Preparation—Peripheral blood mononuclear cells were separated from whole blood of healthy volunteers on a discontinuous Ficoll-Paque gradient (Amersham Pharmacia Biotech, Austria) and cultured immediately in RPMI 1640 medium supplemented with 10% fetal calf serum. In case of activation, PHA was added to cultures at a final concentration of 1 μg/ml for 12 h. HeLa cells were maintained in monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum. Cytoplasmic fractions were prepared as described elsewhere (25) with minor modifications. Briefly, 2–3 $\times$ 10^7 cells were washed in ice-cold serum-free medium, and pellets were resuspended in buffer A containing 10 mM PIPES, pH 6.8, 100 mM KCl, 2.5 mM $MgCl_2$, 300 mM sucrose, 1 mM phenylmethylsulfonyl fluoride and lysed on ice for 3 min with the addition of 1% Triton X-100. Following centrifugation for 3 min at 900 $\times$ *g*, supernatants were collected and frozen immediately until use.

In Vitro RNA Transcription and Label Transfer Studies—The 350-nucleotide-long IFN-γ 3′-UTR and 270-nucleotide-long IL-2 3′-UTR RNAs were transcribed from linearized plasmids containing the 3′-UTR AU-rich instability determinant sequences (ARE) of IFN-γ and IL-2 lymphokine mRNAs as described elsewhere (26, 27). The plasmid pRK5-myc containing the 400-nucleotide-long c-*myc* 3′-UTR was a gift of Dr. John Hesketh, and the probe RNA was generated as described elsewhere (28). The pT7/T3α-19-IL-10hu plasmid containing the full-length IL-10 cDNA (ATCC, 68191) was constructed by inserting into the *Bam*HI site of pT7/T3α-19 vector the IL-10 cDNA. Full-length IL-10 RNA (IL-10) or IL-10 RNA lacking the 3′-UTR (IL-10 (−3′-UTR)) was transcribed from the pT7/T3α-19-IL-10hu plasmid linearized with *Sac*I or *Ssp*I, yielding a respective 1580- and 875-nucleotide-long transcript. The 90-nucleotide-long Δ2R1 and Δ2H3 probes were generated from the same DNA template except that Δ2H3 was transcribed in the antisense orientation yielding a 5$\times$ UAAAU repeat sequence, a corresponding sense sequence of which (AUUUA motif in a 5$\times$ tandem repeat) is found within the 3′-UTR of granulocyte-macrophage-colony-stimulating factor mRNA (29). All transcription reactions were performed in the presence of [^{32}P]UTP. Sequences of the "AUUUA"-containing ARE found in these probes are given in Fig. 1. 10^5 cpm RNA probes with specific activity of ~2–5 $\times$ 10^8 cpm/μg (~10 fmol) were incubated with 0.5 μg of recombinant proteins or 5 μg of total lysate proteins in 12 mM HEPES, pH 7.9, 15 mM KCl, 0.2 mM dithiothreitol, 0.2 μg/ml yeast tRNA, and 10% glycerol at 30 °C for 10 min. In experiments where the effects of ATP, homoribopolymers, or peptide substrates were tested, these agents were added to the reaction simultaneously with the RNA probe. In some experiments, the amount of trace magnesium was increased to 1 mM which did not have a noticeable effect on the extent of RNA binding. Protein-RNA complexes were covalently fixed with ultraviolet light (3000 microwatts/cm^2, 5 min, UV Stratalinker model 1800, Stratagene) on ice and were subsequently RNase-treated (7.5 units of RNase T1 and 15 μg of RNase A/sample) for 15 min at 37 °C. Samples were then separated on 12.5% SDS-PAGE under reducing conditions, and gels were analyzed by autoradiography or PhosphorImaging (Molecular Dynamics).

Immunoprecipitation—Protein A-Sepharose was conjugated with either a monoclonal antibody against human Hsp70 or control antibody (P3, parent hybridoma supernatant). Beads were washed with buffer IP (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF, and 1 μg/ml pepstatin A). 10 μg of total cytoplasmic lysate proteins were incubated with 3 $\times$ 10^5 cpm of ^{32}P-labeled RNA probes, UV cross-linked, and digested with RNases as described above. Immunoprecipitation of RNA-protein complexes was performed in buffer IP at 4 °C by incubating the reaction mixture with the prepared beads for 45 min under gentle agitation. Beads were then pelleted, and the supernatants were collected (depleted fractions). After washing extensively with buffer IP, beads and depleted fractions were analyzed by 12.5% SDS-PAGE and subsequent autoradiography.

RESULTS

Hsp70 and Hsp110 Binds AU-rich RNA in Vitro—Two independent lines of experimental observations turned our attention to examine *in vitro* RNA binding by Hsp70 proteins. First, during examination of RNA binding properties of various deletion mutants of AU-rich RNA-binding fusion proteins, an intense 70-kDa RNA binding activity was co-purifying with these proteins from bacterial extracts (data not shown). Second, our earlier label transfer studies showed that, among others, a 70-kDa complex was frequently detected using cytoplasmic lysates of human lymphocytes and other cell types with AU-rich 3′-UTR RNA sequences of various lymphokine and proto-oncogene mRNAs (see *e.g.* in Ref. 23).

Therefore, to test the possibility that mammalian Hsp70 proteins are capable of binding to AU-rich RNA *in vitro*, recombinant Hsp70 was analyzed in a label transfer assay using [^{32}P]UTP-labeled 3′-UTR RNA probes derived from various lymphokine as well as c-*myc* mRNAs. Fig. 1 illustrates the ARE sequences of the RNA probes used in this analysis. Fig. 2*A*

IFN-γ ARE 5`UCU**AUUUA**UUAAU**AUUUA**ACAUU**AUUUA**UAUAUGGG3`

IL-2 ARE 5`CUCU**AUUUAUUUA**AAAU**AUUUA**ACUUU**AAUUUA**UUUUUGG
AUGUAUUGUUUACUAUCUUUUAGUGCUUCCCACUUAAAAC
AUAUCAGGCUUCU**AUUUAUUUA**AAAU**AUUUA**AAUUUUA
U**AUUUA**UU3`

***c-myc* ARE** 5`AUAAACCCUAAUUUUUUUU**AUUUA**AGUACAUUUUG
CUUUUAAAGUU3`

IL-10 ARE 5`UAGAAU**AUUUA**UUACCUCUGAUACCUCAACCCCCAUUUCU
AUUUAUUUACUGAGCUUCUCUGUGAACG**AUUUA**GAAAGAAG
CCCAAUAUUAUAAUUUUUUUCAAU**AUUUA**UUAUUUUCA3`

Δ2R1 (GM-CSF ARE) 5`AU**AUUUAUUUAUUUAUUUAUUUA**AGUU3`

Δ2H3 (antisense Δ2R1) 5`UAUAAAUAAAUAAAUAAAUAAAUUCAA3`

FIG. 1. **Sequences of the 3′-UTR AU-rich instability determinant elements of the various RNA probes used in this study.** Characteristic AUUUA pentamers are in *boldface*. Linearized plasmid templates harboring these ARE sequences were used to generate [^{32}P]UTP-labeled RNA probes in *in vitro* transcription reactions. Length of each probe RNA is indicated under "Experimental Procedures."

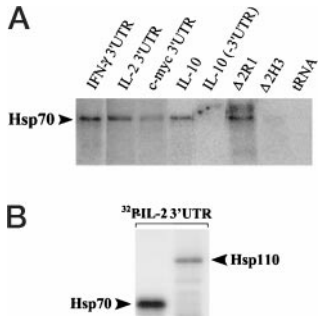

FIG. 2. **AU-rich RNA binding by Hsp70 and Hsp110 proteins.** *A,* recombinant human Hsp70 protein was analyzed in a label transfer assay using various AU-rich RNA probes (see Fig. 1). 0.5 μg of protein was incubated with [^{32}P]UTP-labeled RNA, exposed to monochromatic UV light, and digested with RNase A and T1. RNA-protein complexes were analyzed by 12.5% SDS-PAGE and autoradiography. 3′-UTR-less IL-10 RNA, Δ2H3, and tRNA were used as negative controls. *B,* equal amounts (0.5 μg) of recombinant human Hsp70 and hamster Hsp110 proteins were analyzed in a label transfer assay, using IL-2 3′-UTR RNA probe (indicated at the *top*) as described above.

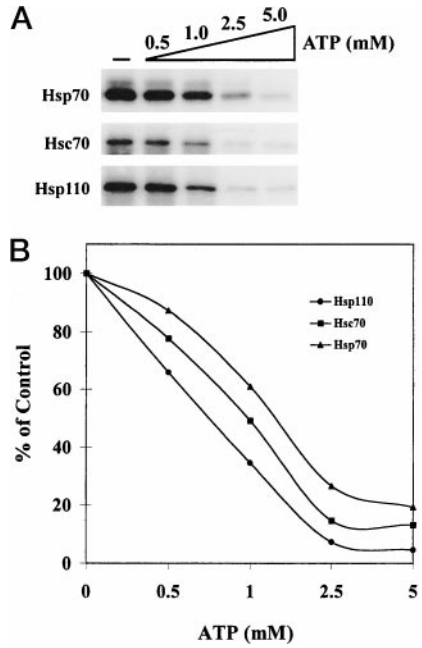

FIG. 3. **Inhibition of RNA binding activity of Hsp70, Hsc70, and Hsp110 proteins by ATP.** *A,* 0.5 μg of total proteins was analyzed for IL-2 3′-UTR RNA binding in the presence of various concentrations of ATP as indicated at the *top of the panel.* Following UV-cross-linking and RNase digestion, complexes were analyzed by SDS-PAGE and PhosphorImaging. *B,* PhosphorImage values for each band were expressed and plotted as percent of controls, where no ATP was present in the RNA-binding reaction.

suggests that Hsp70 binds to these various AU-rich RNA sequences with a comparable intensity (*1st 3 lanes*). RNA binding is restricted to the 3′-UTR and is not likely to involve open reading frame sequences as Hsp70 binds to the full-length IL-10 probe but not to IL-10 RNA without the 3′-UTR (*4th* and *5th lanes*). To ensure that Hsp binding required the presence of the AUUUA motifs and not the AU context *per se*, Δ2R1, which encodes the 90-nucleotide-long ARE of granulocyte-macrophage-colony-stimulating factor with five reiterated AUUUA pentamers and Δ2H3, its antisense sequence with five "UAAAU" motifs were compared. Whereas Δ2R1 displayed comparable Hsp70 binding relative to the other ARE probes, Δ2H3 was similar to tRNA in that they are not recognized by Hsp70 (*lanes 6–8*). These data strongly suggest that, *in vitro*, Hsp70 preferentially binds AU-rich 3′-UTR sequences common to many unstable mRNAs.

Since Hsp110 is a unique and recently characterized member of the Hsp70 superfamily, its ability to bind RNA was also examined. By sequence analysis and molecular graphics, Hsp110 has been shown to contain the primary elements common to other members of the Hsp70 family of proteins (12, 13). Fig. 2*B* demonstrates that, like Hsp70, recombinant Hsp110 also binds the AU-rich 3′-UTR RNA sequences of the IL-2 mRNA but not tRNA (data not shown). This suggests that RNA binding might be associated with a region that is common to and homologous in the two proteins. However, it is also seen that despite comparable molar ratios of protein and RNA in the reaction, the binding activity of Hsp110 is notably weaker than that of Hsp70. This represents a qualitative difference between members of these two related, but distinct, stress protein families.

ATP Modulates in Vitro RNA Binding by Hsp/Hsc70 and Hsp110—Previous studies have shown that the di-nucleotide-binding domain (also called the Rossmann fold) of glyceraldehyde-3-phosphate dehydrogenase represents an RNA-binding motif and that NAD^+ or NADH as well as ATP were effective in modulating *in vitro* RNA binding by this metabolic enzyme (26).[2]foot;f2;10> Since ATP is also an important modulator of Hsp70 function through its association with the N-terminal ATP-binding motif in this protein, we examined the possibility whether ATP had a modulatory effect on AU-rich RNA binding by Hsps. Fig. 3 illustrates the result of this study. It is seen that RNA binding by Hsp70, Hsc70, and Hsp110 proteins was substantially reduced by ATP in the 0.5–5 mM range. The autoradiogram shown for Hsp110 required an approximately 4× exposure to provide a signal intensity comparable to those of Hsp70 and Hsc70. It is also seen that the half-maximal RNA binding by all three proteins was observed within the physiological ATP concentration range (~1 mM, Fig. 3*B*). The presence of Mg^{2+} in the RNA-binding reaction did not have any noticeable effect on the ATP modulation of Hsp RNA binding (data not shown). These results indicate that ATP is a common and potent modulator of RNA binding by both Hsp/Hsc70 and Hsp110 proteins and that the ATP-binding domain common to these proteins may also be involved in RNA binding.

Binding of RNA to Targeted Deletion Mutants of Hsp110—To show a direct involvement of the ATP-binding domain in RNA binding, deletion mutants of Hsp110 that contain different predicted functional domains were assessed for RNA binding in label transfer assays. The regions indicated have been determined by comparative sequence alignment and molecular graphics using DnaK as a model (12, 31). Briefly, Hsp110 contains basic ATP-binding sequences (residues 0–375), a pre-

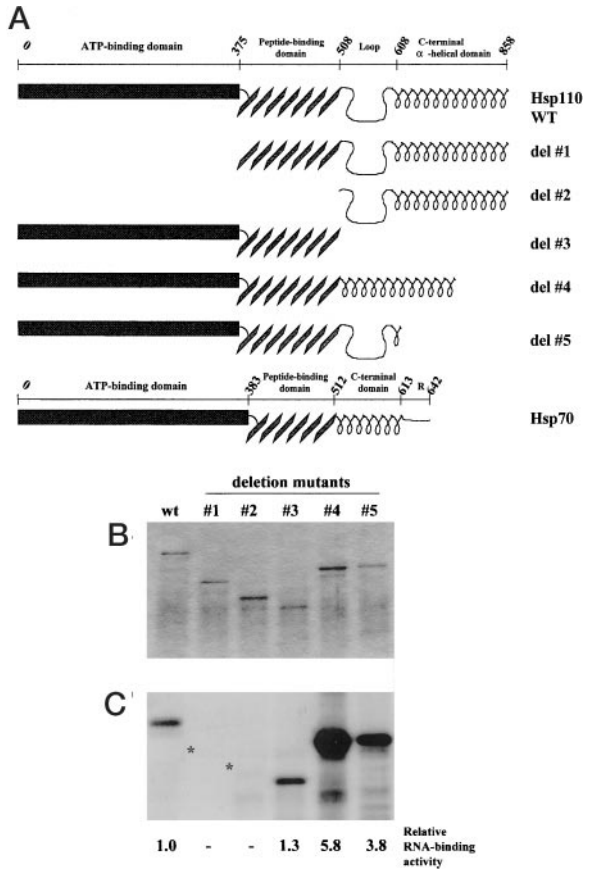

FIG. 4. *A,* schematic illustration of domain structure of wild type (*wt*) Hsp110 and its various deletion mutants. Amino acid positions of each structural domain are given at the *top of the panel*. A unique structural feature of Hsp110 is a 100-amino acid-long (amino acids 508–608) α-loop structure, not present in any other members of the Hsp70 family. Domain structure of Hsp70 and proportional length of each domain relative to Hsp110 is given at the *bottom of the panel*. *R* indicates the most C-terminal regulatory domain of Hsp70. *B,* Coomassie Brillant Blue-stained gel of the label transfer assay shown in *C* illustrates size differences of wild type (*lane 1*) and deletion mutants 1–5 (*2nd to 6th lanes*, respectively). This gel served as a loading control for the evaluation of relative RNA binding intensities of each protein. *C,* wild type (*1st lane*) and all Hsp110 deletion mutants (*del 1–5* in *2nd to 6th lanes*, respectively) were analyzed for RNA binding in a label transfer assay using the IL-2 3′-UTR RNA probe. Relative intensities of RNA binding measured by densitometric analysis of each bands are shown *below the corresponding lanes* and expressed relative to that of the wild type which was chosen 1. *Asterisks* indicate the positions of deletion mutants *#1* and *#2* as verified from the alignment of the autoradiogram with the Coomassie-stained gel.

dicted peptide-binding domain (residues 375–508), a loop domain (residues 508–608), and a predicted series of α-helices making up its C terminus (residues 608–858). The domain composition of wild type Hsp110 (*WT*) and mutants 1–5 as well as of Hsp70 are presented schematically in Fig. 4*A*. These mutants and their ability to exhibit chaperoning functions have been described elsewhere (24). The RNA binding experiments revealed that the N-terminal 375-amino acid-long ATP-binding domain is required for RNA binding based on the observation that deletion mutants 1 and 2, which lack the ATP-binding domain, showed no RNA binding activity (Fig. 4*C*). Interestingly, these experiments also demonstrated that removal of the 100-amino acid-long α-loop structure (mutant 4) or the C-terminal α-helical domain (mutant 5) of Hsp110 further enhanced its ability to bind RNA, by 5.8- and 3.8-fold, respectively (Fig. 4*C*), *i.e.* making it more comparable in RNA binding to that seen for Hsp70 in Fig. 2*C*. RNA binding activity by deletion mutant 3, in which both of these distal elements were deleted,

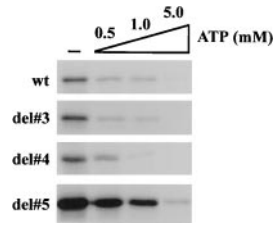

FIG. 5. **Effect of ATP on *in vitro* RNA binding by wild type and mutant Hsp110 proteins.** 0.5 μg total of each protein was analyzed in a label transfer experiment using IL-2 3′-UTR RNA probe in the presence of ATP as indicated at the *top of the panel*. RNA-protein complexes were analyzed using SDS-PAGE and autoradiography.

was only marginally increased (by 1.3-fold) relative to that of wild type Hsp110 (Fig. 4*C*). These data, nevertheless, further illustrate direct RNA binding by these heat shock proteins.

To characterize further binding of Hsp110 deletion mutant proteins to AU-rich RNA, we also examined the effect of ATP on this parameter. Similar to that of wild type Hsp110, ATP was capable of diminishing *in vitro* RNA binding by all three mutants (*del 3–5,* Fig. 5). Similar to the studies on wild type Hsp110 and Hsp70, an ATP concentration of 1 mM largely abrogated RNA binding of all proteins which demonstrates that regardless of the presence of downstream C-terminal portions of the molecule, RNA binding by the N-terminal domain is still sensitive to and regulated by ATP.

We also examined the competition of homoribopolymers on RNA binding by Hsp110 and its deletion mutants and found that AU-rich RNA binding by all three mutant proteins, but not that by the wild type Hsp110, was markedly diminished by poly(U) (data not shown). Whereas no effect of poly(A) or poly(C) was evident on RNA binding by either proteins, interestingly, poly(G) was a strong competitor of AU-rich RNA binding by both the wild type and truncated proteins (data not shown) (also see "Discussion").

Peptide Substrate Interferes with RNA Binding by Hsp70 and Hsp110 Proteins—The most noticeable function of Hsps under physiological conditions is the recognition and binding of misfolded or partially unfolded regions of polypeptide chains. Since the N-terminal ATP-binding domain and the C-terminal peptide-binding region have been suggested to interact through the transmission of conformational changes (15, 31–35), we assessed whether peptide binding is capable of modulating or interfering with RNA binding by Hsps *in vitro*. For this purpose, we introduced reduced carboxymethylated lactalbumin (RCMLA) into the binding reaction. This fully extended, nonnative conformational homologue of α-lactalbumin has been shown to be an effective peptide substrate of Hsp/Hsc70 (36). Fig. 6 illustrates that even at a 1:1 molar ratio for Hsp70 or a 1:6 molar ratio for Hsp110 (0.1 μg of RCMLA), peptide substrate reduced RNA binding by ~20–25%. RCMLA, when examined in a similar assay, did not bind RNA (data not shown). Peptide substrate binding by these Hsps is not affected by the buffer used in the RNA binding assay as prevention of thermal aggregation of luciferase by both Hsp70 and Hsp110 proteins is comparable in this buffer to that obtained in the usual aggregation buffer (data not shown). These experiments indicated that peptide binding by either Hsp70 or Hsp110 chaperones interferes with their RNA binding.

Immunoprecipitation of AU-rich RNA-Protein Complexes by Anti-Hsp70 Antibody—Many regulatory steps in the metabolism of unstable mRNAs have been implicated to occur via interaction of ARE with various protein factors (AUBP) (see *e.g.* Refs. 37 and 38). Formation and/or dissociation of specific ARE·AUBP complexes might be mediated by additional components as shown with certain cytoskeletal elements (39). Be-

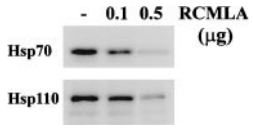

FIG. 6. **Peptide substrate binding modifies *in vitro* RNA binding activity of Hsp70 and Hsp110 proteins.** RCMLA was added to the label transfer reaction at the indicated concentrations, in which 0.5 μg of Hsp70 or Hsp110 proteins were analyzed for binding to IL-2 3′-UTR RNA probe. RNA-protein complexes were analyzed by SDS-PAGE and autoradiography.

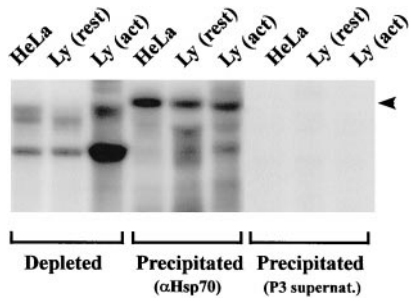

FIG. 7. **Immunoprecipitation of RNA-protein complexes by anti-Hsp70 antibody.** Equal amounts of total cytoplasmic proteins of HeLa and resting (*rest*) or PHA-activated (*act*) human lymphocytes were incubated with radiolabeled IFN-γ 3′-UTR RNA probe as described. Reactions were subsequently immunoprecipitated as described under "Experimental Procedures," and proportional aliquots of immunodepleted and precipitated fractions were analyzed by 12.5% SDS-PAGE and autoradiography. *Arrowhead* indicates the position of a 70-kDa complex that was specifically immunoprecipitated from each cell fraction.

cause of the demonstrated affinity of members of the Hsp70 family of proteins to ARE and to answer the question as to whether Hsp70 proteins might potentially be part of an ARE·AUBP complex, we used a direct immunoprecipitation approach. As previously demonstrated, RNA binding activity by many AUBPs is markedly up-regulated upon activation of lymphocytes by a variety of stimuli (see *e.g.* Ref. 40). Therefore, cytoplasmic extracts of both resting and PHA-activated human lymphocytes as well as HeLa cells were prepared as described under "Experimental Procedures," and equal amounts of total proteins were incubated with IFN-γ mRNA 3′-UTR probe. RNA-protein complexes were UV cross-linked and exposed to RNase treatment. Prior to SDS-PAGE, reactions were immunoprecipitated using monoclonal antibodies against human Hsp70. Fig. 7 illustrates a result of a typical experiment and shows that a 70-kDa complex immunoprecipitates from all three label transfer reactions. Fig. 7 also shows that, in accordance with our previous findings, it is the activity of a 36-kDa complex that is most notably increased upon lymphocyte activation. No radioactive signal could be detected in the precipitated fraction in any of multiple immunoprecipitation assays using P3 supernatant as a negative control. Occasionally we observed additional, less intense bands in the precipitated fractions. Although we think these represent degradation products of the complex which still retain some of the labeled RNA probe, the possibility that they arise from other AUBPs entrapped in Hsp·RNA complexes and released during denaturation cannot be ruled out. Nevertheless, these experiments demonstrate that AU-rich 3′-UTR RNA probes are recognized and bound *in vitro* by endogenous Hsp70, *i.e.* proteins derived from different mammalian cell types. Thus, these results indicate that the formation of Hsp70·ARE complexes may also occur in the cell.

DISCUSSION

The 70-kDa heat shock protein family has received considerable attention in the past decade. Recognition of members of the Hsp70 family as molecular constituents in a wide range of important cellular mechanisms (reviewed in Refs. 2 and 41) has long emphasized the universal importance of these proteins in cell physiology. In this report we extend these findings by providing evidence for direct *in vitro* RNA binding by members of the mammalian Hsp70 protein family, a feature that has not previously been described. We demonstrate that both the stress-inducible (Hsp70) and cognate (Hsc70) forms as well as Hsp110, the distantly related member of the mammalian Hsp70 family, bind AU-rich 3′-UTR RNA sequences that function as instability determinants of various lymphokine and proto-oncogene mRNAs. By using a series of deletion mutants of Hsp110, we were able to localize RNA binding to the N-terminal ATP-binding domain. This finding was strengthened by the capability of ATP to reduce RNA binding *in vitro* at physiological ATP concentrations (1–2 mM). The ATP concentrations required for noticeable competition with RNA binding is considerably higher than the K_m value reported for Hsp70 and related proteins with chaperone ATPase activity (K_m $\sim 10^{-6}$ M, Ref. 31). This suggests that RNA binding by the N terminus of the chaperone molecule involves other, presumably larger regions within the ATP-binding domain than those responsible for the binding of ATP. This view is also consistent with our previous finding that actin, whose structure and folding topology are nearly identical to that of the ATPase fragment of Hsc70 (32), does not bind RNA (39).

We also concluded that the C-terminal regions of Hsp110 can influence the interaction with RNA based on the following observations. First, removal (mutants 3 and 5) or rearrangement (mutant 4) of the C-terminal portion of Hsp110 considerably increased the RNA binding activity of the protein. ATP sensitivity of RNA binding by these mutant proteins was retained, suggesting that the ATP-binding domain is involved in a way similar to that of the wild type Hsp110. Second, engagement of the C-terminal peptide-binding domain by a substrate paralleled with considerable decrease in RNA binding activity (Fig. 6). Although in light of the present results one can only speculate how RNA binding is influenced by C-terminal structures, these data appear to be consistent with the view that intramolecular interactions between the ATP-binding and peptide-binding domains trigger conformational rearrangements within the chaperone molecule. Such conformational changes have been suggested to elicit the transduction of important information in chaperoning function (31–35). The functional role of the 100-amino acid-long α-loop structure unique to Hsp110 is not known. It is mechanistically possible that this unit may serve as a 'connector' between the large N-terminal region harboring both the ATP- and peptide-binding domains and the C-terminal lid structure. This putative function would allow flexible communication of the C terminus with the domains that mediate ATP and peptide binding. In deletion mutants, this putative interaction may be impaired due to the lack of the loop (mutant 4), the C terminus (mutant 5), or both (mutant 3). Therefore, the ATP-binding domain, responsible for RNA binding, may become simply more exposed or it may take up a different conformation favored by RNA recognition and binding.

We observed that in all cases, poly(G) appeared to be the strongest competitor of RNA binding. It may well be that under the *in vitro* conditions used, oligo(G) stretches are the structural preference for these proteins to bind to, but since such sequence is not likely to exist *in vivo*, this interaction *per se* may not be a major element of possible *in vivo* function. The

observed interaction of Hsp70 with poly(G) is in good agreement with the earlier observations obtained with *Drosophila* Hsp70 using a homoribopolymer affinity chromatographic approach.[3] The U preference, however, may be of considerable importance as long U stretches as well as U-rich RNA sequences are featured in many determinants of mRNA stability, including ARE (38). The question remains, however, what is the physiological relevance of AU-rich RNA binding by Hsps? The finding that Hsp70 can be immunoprecipitated from a label transfer reaction of RNA-protein complexes that had been formed between AU-rich RNA probes and cytoplasmic lysates of lymphocytes or HeLa cells (Fig. 7) suggests that Hsp70 may potentially associate with such (and perhaps many other) RNA entities *in vivo*.

One clearly relevant observation that links Hsp70 proteins to activation-related lymphokine mRNA metabolism is work by Di *et al.* (43) who have established that Hsp70 translocates into a cytoplasmic spectrin-based aggregate upon lymphocyte activation and that this association is abolished by ATP both *in vitro* and *in vivo* (42). It is possible that the activation-induced formation of the large cytoplasmic spectrin aggregate is in favor of providing a preferential cytoplasmic environment where regulatory mechanisms of mRNA stability and translation take place. Hsp70 and likely other chaperones may be recruited into this compartment to facilitate, through direct RNA binding, the unwinding of complex secondary structures. This would aid in the exposure of critical *cis*-acting sequences for other protein factors to bind and, hence, allow proper and efficient assembly of polysomes or complexes involved in mRNA degradation. Such "nucleic acid chaperone" function had been designated to other non-chaperone proteins, such as nucleocapsid proteins of RNA viruses (44), heteronuclear ribonucleoprotein A1 (40, 45), and glyceraldehyde-3-phosphate dehydrogenase (26).[2]

Alternatively, Hsp70 and Hsp110 may also function to modulate the interaction of a given mRNA with regulatory proteins that would influence mRNA stability and/or translation (49). In support of this, recently Scandurro and co-workers (46) have suggested that Hsp70 is a potent regulator of complex formation between the 3′-UTR of erythropoietin mRNA and its specific binding protein, erythropoietin mRNA-binding protein. Assembly of this complex is necessary for hypoxia-induced stabilization of erythropoietin mRNA. Under normoxic conditions, when stress-protective chaperoning activity of Hsp70 is not operant, this protein may sequester erythropoieton mRNA-binding protein to prevent its binding to erythropoietin mRNA and the mRNA is being degraded (46). Moreover, it has recently been shown that evolutionarily highly conserved regions identified through extensive data base screening overlap with AU-rich sequences with known function in post-transcriptional regulatory mechanisms (30). Using a modular retroviral vector, highly conserved regions of various origins were analyzed for post-transcriptional regulatory activity under different conditions *in vivo*. Results from these studies allowed the conclusion that some highly conserved regions may function as stress sensor elements to promote rapid adaptation of cells to various stress conditions (30). It is, therefore, feasible to speculate that certain Hsps might also be integral parts of such adaptation/sensor systems at the post-transcriptional level of gene expression.

In conclusion, based on these results, we propose that members of the Hsp70 family, including Hsp110, may directly and specifically participate in molecular events underlying lymphokine mRNA stability and/or translation following lymphocyte activation. Hsps may bind lymphokine mRNAs, mediating an "RNA chaperone" function that might be required for proper folding of the RNA to expose critical motifs involved in regulatory events during translation and/or decay. Alternatively, Hsp70 chaperones may be required in the fine regulation of messenger ribonucleoprotein complex formation with other RNA-binding proteins, through the modulation of their folding state or facilitation of their microcompartmentation. It is also possible that direct protection of RNA and/or stabilization of RNA-protein complexes by chaperones are relevant mechanisms during various stress conditions.

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[3] Susan Lindquist, personal communication.

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