Differential Inhibition of Hsc70 Activities by Two Hsc70-Binding Peptides†

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ABSTRACT: The ability of two high-affinity Hsc70-binding peptides [FYQLALT (peptide-Φ) and NIVRKKK (peptide-K)] to differentially inhibit Hsc70-dependent processes in rabbit reticulocyte lysate (RRL) was examined. Both peptide-Φ and peptide-K inhibited chaperone-dependent renaturation of luciferase in RRL. Peptide-Φ, but not peptide-K, blocked Hsp90/Hsc70-dependent transformation of the heme-regulated eIF2α kinase (HRI) into an active, heme-regulatable kinase. In contrast, peptide-K, but not peptide-Φ, inhibited Hsc70-mediated suppression of the activation of mature-transformed HRI. Furthermore, HDJ2 (Human DnaJ homologue 2), but not HDJ1, potentiated the ability of Hsc70 to suppress the activation of HRI in RRL. Mechanistically, peptide-K inhibited, while peptide-Φ enhanced, HDJ2-induced stimulation of Hsc70 ATPase activity in vitro. The data presented support the hypotheses that peptide-Φ acts to inhibit Hsc70 function by binding to the hydrophobic peptide-binding cleft of Hsc70, while peptide-K acts through binding to a site that modulates the interaction of Hsc70 with DnaJ homologues. Overall, the data indicate that peptide-Φ and peptide-K have differential effects on Hsc70 functions under quasi-physiological conditions in RRL, and suggest that therapeutically valuable peptide mimetics can be designed to inhibit specific functions of Hsc70.

Members of the Hsp70[1] family of molecular chaperones have diverse biochemical roles in cells including: modulating the assembly and disassembly of protein complexes; facilitating protein renaturation; preventing aggregation of denatured proteins; and chaperoning the transport and folding of newly translated polypeptides in the cytosol and within organelles [reviewed in (*1*−*3*)]. The functions of Hsp70 family members are modulated through their interactions with other cochaperones and cohorts, such as DnaJ homologues (*4*−*10*), p48/HIP (*11*−*13*), p60/HOP (*13*−*15*), and BAG-1 (*16*, *17*). DnaJ homologues stimulate the ATPase activity of Hsc70 (*4*, *7*−*10*), but also have domains that specifically interact with protein clients (*5*, *6*, *18*, *19*). BAG-1, which appears to be the eukaryotic equivalent of GrpE, stimulates the exchange of Hsc70-bound ADP for ATP and the release of Hsc70-bound client proteins (*20*−*24*). p48/HIP stabilizes the ADP-bound form of Hsc70 (*12*, *13*, *25*), while p60/HOP (Hsp70−Hsp90 organizing protein) modulates the interaction of Hsc70 with the Hsp90 superchaperone complex (*13*, *14*).

Screening of phage display libraries has defined two different but perhaps overlapping binding specificities for Hsc70 (*26*). Hsc70 can bind with high affinity to peptides that are primarily composed of alternating large hydrophobic (Φ) amino acids (e.g., peptide-Φ, FYQLALT) or to peptides containing hydrophobic amino acids followed by a run of basic amino acids (e.g., peptide-K; NIVRKKK) (*26*). High-affinity binding of Hsp70s to hydrophobic peptides, such as peptide-Φ, requires that the peptide be at least seven amino acid residues long and in an extended conformation (*27*−*29*). Hydrophobic peptides such as peptide-Φ stimulate the ATPase activity of Hsc70, while peptide-K does not (*4*, *19*, *26*). Hsc70 molecules containing bound peptide-Φ have faster electrophoretic mobility in native polyacrylamide gels than free Hsc70, with free Hsc70 having a faster electrophoretic mobility than Hsc70 containing bound peptide-K (*26*). Thus, peptide-Φ and peptide-K appear to interact with distinct conformations of Hsc70.

In this report, we have examined whether peptide-Φ and peptide-K have differential effects on Hsc70 functions in RRL, as agents that bind to distinct conformations of Hsc70 would be predicted to differentially disrupt the normal interactions of Hsc70 with client substrates, cohorts, or cochaperones. We examined the effects of peptide-Φ and peptide-K on: the renaturation of denatured protein (e.g., firefly luciferase); Hsp90/Hsc70-dependent maturation of protein kinases; Hsc70-mediated attenuation of the activation of the heme-regulated eIF2α kinase; and DnaJ-induced

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[1] Abbreviations: HRI, heme-regulated inhibitor (heme-regulated eIF2α kinase); eIF, eukaryotic initiation factor; eIF2α, α-subunit of eIF2; NT-HBD, heme-binding domain of HRI; RRL, rabbit reticulocyte lysate; TnT RRL, nuclease-treated reticulocyte lysate with coupled transcription and translation; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; VIEF, vertical isoelectric focusing; TBS, Tris-buffered saline [buffer containing 20 mM Tris·HCl (pH 7.4) and 150 mM NaCl]; PVDF, poly(vinylidene difluoride); peptide-Φ, FYQLALT; peptide-K, NIVRKKK; HDJ, Human DnaJ homologue; Hsc70, 70 kDa heat shock cognate protein; Hsp, heat shock protein; RCM-BSA, reduced, carboxymethylated bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; DMSO, dimethyl sulfoxide; BAHE, bromoacetic acid *N*-hydroxysuccinimide ester; GaG-agarose, goat anti-mouse IgG cross-linked to agarose; ATA, aurintricarboxylic acid; SH3, Src-homology domain 3.

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stimulation of the ATPase activity of Hsc70. The data indicate that peptide-Φ and peptide-K indeed have differential effects on Hsc70 functions in RRL, and suggest that peptide mimetics can be designed to inhibit specific functions of Hsc70.

MATERIALS AND METHODS

Materials. Bovine serum albumin (acetylated), luciferin, and luciferase were obtained from Sigma. L-[^{14}C]-Leucine and L-[^{35}S]-methionine were purchased from DuPont-NEN. TnT-coupled reticulocyte lysate and affinity-purified anti-luciferase antibody were obtained from Promega. Geldanamycin (GA) was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Anti-His-tag (His_5) antibody was purchased from Qiagen. The BB70 anti-Hsp70 and purified HDJ2 were provided by David Toft (Mayo Medical School, Rochester, MN). The anti-eIF2α monoclonal antibodies were provided by Edgar Henshaw (University of Rochester, Syracuse, NY). N27 anti-Hsp70 mAb was purchased from StressGen. Anti-DnaJ antibody and purified HDJ1 were provided by William Welch (University of California, San Francisco). Hsc70 was purified as previously described (*30*).

Buffers. The buffers used were as follows: Assay buffer (AB) consisted of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 470 μM D-luciferin, 240 μM coenzyme A, and 0.5 mM ATP; stability buffer (SB) consisted of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, 10 mg/mL bovine serum albumin, 10% glycerol, and 1% Triton X-100; Tris buffer (TB) consisted of 10 mM Tris-HCl (pH 7.4); Tris-buffered saline (TBS) consisted of TB and 150 mM NaCl; TB/500 consisted of TB and 500 mM NaCl and TB/50 consisted of TB and 50 mM KCl; TBS detergent buffer (TBSD) consisted of TBS and 1% deoxycholate, 1% Triton X-100, and 0.1% SDS.

Luciferase Renaturation Assay. The effect of agents on the ability of RRL to renature luciferase was assayed as previously described (*31*−*33*). Briefly, firefly luciferase (0.5 mg/mL) in SB was denatured by incubation at 42 °C for 10 min (*31*). After being cooled on ice, aliquots were diluted 20-fold into heme-deficient protein synthesis mixes containing 10 mM creatine phosphate, 20 units/mL creatine phosphokinase. Samples were incubated at 28 °C for 10 min; luciferase activity present in 1.0 μL aliquots was determined by dilution into 50 μL of AB. Light production was measured for 10 s in a Lumac (3M) Bioluminometer.

Coimmunoadsorption of Proteins Associated with Denatured Luciferase. Affinity-purified anti-luciferase antibody (1 μL) was adsorbed onto 20 μL of anti-rabbit IgG-agarose in the presence or absence (control) of luciferase (10 μg) for 2 h on ice as previously described (*33*, *34*). Heme-deficient protein synthesis mixes (30 μL), which had been incubated for 10 min at 30 °C with peptides or vehicle control (DMSO), were added to the agarose pellets. The mixtures were incubated for 10 min at 30 °C with periodic mixing to prevent settling of the agarose. The unadsorbed proteins were separated from adsorbed proteins (pellets) by centrifugation. Pellets were washed with 750 μL of TB/50, 3 times with TBSD, and once with TB/50. Samples were then analyzed by SDS−PAGE and western blotting as described previously.

Cross-Linking Peptide-K to Agarose. Aminoethyl-Sepharose in 2 mL of 0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA was incubated with bromoacetic acid *N*-hydroxysuccinimide ester (BAHE) (34 mg dissolved in 0.25 mL of dimethylformamide) at 4 °C for 2 h. The BAHE-activated resin was then washed with ice-cold 0.1 M NaCl, followed by washing with 0.1 M sodium phosphate buffer (pH 7) containing 1 mM EDTA. Approximately 10 μmol of peptide-K containing a spacer of three Gly residues and a Cys residue at its C-terminus (peptide-KC: NIVRKKKGGGC) was then cross-linked through the Cys thiol to the BAHE-activated aminoethyl-Sepharose at room temperature for 4 h with continuous shaking, followed by incubation with 0.2 M 2-mercaptoethanol overnight to block any unreacted groups. A negative control resin (NCR) for peptide-KC-Sepharose was prepared by reacting BAHE-activated aminoethyl-Sepharose with 0.2 M β-mercaptoethanol. The resins were washed with and stored in 0.1 M sodium phosphate buffer (pH 7) containing 1 mM EDTA.

Affinity Adsorption with Peptide-K-Sepharose. RRL was incubated under protein synthesis conditions at 30 °C for 20 min with or without addition of peptide-K or peptide-Φ. Peptide-KC-Sepharose (8 μL) or NCR was then added to 16 μL of protein synthesis mixes, and incubation at 30 °C was continued for an additional 10 min. The resin was then washed 5 times with TB/50, boiled in 1× SDS sample buffer for 5 min, and analyzed by 10% SDS−PAGE and western blotting.

Effects of Peptide-Φ and Peptide-K on HDJ2-Induced Stimulation of Hsc70 ATPase Activity. ATPase assays were performed for 25 min at 33 °C in the presence of [α-^{32}P]-ATP (10 μCi), 20 μM ATP, 50 mM pH 7.4 Tris-HCl, 2 mM $MgCl_2$, and 5 mM dithiothreitol. HDJ2 (0.25 μg/μL) and Hsc70 (1 μg/μL) were used for each assay. DMSO and water were used as the vehicle controls for experiments carried out with peptide-Φ and peptide-K, respectively. Each sample was spotted on polyethylenimine cellulose (Sigma), and then thin-layer chromatography was developed in 2 mM formic acid and 0.5 M LiCl to separate ATP and ADP. Radioactivity was quantified by phosphorimaging using a Bio-Rad GS-525 Molecular Imaging System.

De Novo Synthesis and Maturation of HRI. Coupled transcription/translation of HRI and (His_7)HRI was initiated in nuclease-treated rabbit reticulocyte lysate (TnT RRL, Promega) at 30 °C in the absence of [^{35}S]-Met for 15 min as described previously (*35*, *36*). At 15 min, a pulse of 460 μCi/mL [^{35}S]-Met was given. After 4 min of radiolabeling, 1 volume of TnT protein synthesis mix containing [^{35}S]-Met-labeled HRI {[^{35}S]-(His_7)HRI} was mixed with 7 volumes of normal hemin-supplemented (10 μM hemin) or heme-deficient protein synthesis mixes containing non-nuclease-treated RRL and the protein synthesis initiation inhibitor aurintricarboxylic acid (ATA, 60 μM). [^{35}S]-(His_7)-HRI was then incubated for 60 min at 30 °C to yield mature-competent HRI (plus heme RRL) or transformed HRI (minus heme RRL), or for 45 min in heme-deficient RRL followed by 15 min incubation in the presence of 10 μM hemin (repressed HRI) (*35*, *36*). Experiments utilizing HRI generated de novo via TnT were carried out with incubation times varying from 60 to 90 min, as approximately 45 min is required to obtain the maximum amount of transformation of HRI in heme-deficient RRL (*35*, *36*).

Assay of the HRI and p56^{lck} Kinase Activity. RRL mixes containing [^{35}S]-(His$_7$)HRI or [^{35}S]-HRI as a control for nonspecific binding were clarified by centrifugation at 10 000 rpm for 5 min before adsorption to immobilized anti-His-tag antibody as described (*37*). [^{35}S]-(His$_7$)HRI from 25 μL of RRL reaction mixes was bound to anti-His-tag antibody resin (10 μL) for 1 h on ice, followed by 5 washes with 500 μL of buffer containing 50 mM Tris-HCl (pH 7.5) and 50 mM NaCl. Assays for the kinase activity of [^{35}S]-(His$_7$)HRI bound to resin were performed for 4 min at 30 °C as described (*35, 36*). Samples were analyzed by 10% SDS−PAGE, followed by transfer to a PVDF membrane and autoradiography as described previously (*38*). Autophosphorylation of HRI was assayed by the incorporation of [^{32}P]-P$_i$ into HRI during eIF2α kinase assays incubated with [γ-^{32}P]-ATP. ^{32}P-Labeled HRI and eIF2α were detected by quantitatively quenching ^{35}S emissions with three intervening layers of previously developed X-ray film. The kinase activity of His-tagged p56^{lck} generated de novo by coupled transcription/translation in RRL was carried out as previously described (*39, 40*) after immunoadsorption of RRL with immobilized anti-His-tag antibodies, and nonimmune control antibodies (NI) were done as described previously (*37*).

Immunoadsorption of Hsc70. Preparation of GaG-agarose (goat anti-mouse IgG cross-linked to agarose), binding of BB70 anti-Hsc70 monoclonal antibody (or nonimmune control monoclonal antibody), and coimmunoadsorption of HRI with Hsc70 were carried out as previously described (*41, 42*). Clarified RRL mixes (20−25 μL) containing [^{35}S]-HRI were used for immunoadsorption. After 60 min of binding on ice, immunopellets were washed 3 times with 500 μL of wash buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1% Tween-20, and 10 mM monothioglycerol], and the immunopellets were eluted with SDS−PAGE sample buffer. Proteins present in immunopellets and supernatants were separated on 10% SDS−PAGE and transferred to a PVDF membrane. [^{35}S]-HRI was detected by autoradiography.

Preparation of and Immunoadsorptions with Anti-HRI IgY Antibody. Recombinant N-terminal heme-binding domain (NT-HBD) of HRI was purified as previously described (*43*). White leghorn chickens (22−24 weeks old) were immunized subcutaneously with 1 mg of NT-HBD in phosphate-buffered saline (pH 7.2) emulsified in an equal volume (1 mL) of complete Freund's adjuvant. Three booster injections were given on days 12, 24, and 36. Eggs were collected and stored at 4 °C until processing.

Chicken IgY was purified as described previously (*44*). Briefly, yolks were separated from egg whites and yolk membrane, and diluted in 10 volumes of water. The mixture was incubated for 6 h at 4 °C followed by centrifugation at 10000*g* for 25 min at 4 °C. The supernatant was removed and brought to 19% in sodium sulfate. The precipitated IgY was collected by centrifugation at 10000*g* for 25 min at 4 °C, and the IgY was resuspended in TBS and concentrated by ultrafiltration.

Chicken anti-HRI IgY and nonimmune chicken IgY were cross-linked to agarose (CaH-agarose and NIC-agarose) as previously described (*41*). The resin was washed sequentially with TB/50/Tween, TBS, TB/50/Tween, and twice with TBS prior to use. RRL was incubated under protein synthesis conditions at 30 °C for 20 min with or without 5 mM peptide-K or 0.5 mM peptide-Φ. Fifty microliters of protein synthesis mix was then added to washed CaH-agarose or NIC-agarose (20 μL), and HRI was immunoadsorbed by incubation on ice for 90 min. The resin was then washed 3 times with TB/50/Tween, boiled in 1× SDS sample buffer for 5 min, and applied to 10% SDS−PAGE and western-blotted.

Protein Synthesis and eIF-2α Phosphorylation in Reticulocyte Lysates. Protein synthesis was carried out at 30 °C in standard reticulocyte lysate reaction mixtures with or without the addition of radiolabeled amino acid as described (*45, 46*). Hemin-supplemented lysates contained 20 μM hemin-HCl. Protein synthesis was determined by measuring the incorporation of [^{35}S]-methionine or [^{14}C]-leucine into the acid-precipitable protein at 30 °C in standard reticulocyte reaction mixtures. eIF-2α phosphorylations in 2 μL of protein synthesis mixes were analyzed as previously described by western blotting of one-dimensional isoelectric focusing slab gels using 1:1000 dilution of anti-eIF-2α monoclonal ascites fluid (*30, 47, 48*). Protein synthesis in hemin-supplemented RRL remains linear for only 30 min. Therefore, experimental protocols utilizing native RRL were carried out with incubation times between 10 and 30 min, such that changes in eIF2α phosphorylation, eIF2B activity, and protein interactions with endogenous HRI could be directly correlated with rates of protein synthesis.

eIF-2α GTP/GDP Exchange. RRL's were incubated under protein synthesizing conditions at 30 °C in the absence of [^{14}C]-leucine as described (*45, 46*). The rate at which [^{3}H]-GDP was exchanged from preformed eIF-2•[^{3}H]-GDP complexes was measured as described previously (*30, 49*). At the times indicated, 50 μL of PS mix was mixed with 130 μL of ice-cold dilution buffer [40 mM Tris-HCl, pH 7.4, 100 mM KCl, 50 mM KF, 2 mM Mg(OAc)$_2$, 10% glycerol, 40 μM GDP], and 20 μL of preformed eIF2•[^{3}H]-GDP complex. Reactions mixes were then incubated at 30 °C for another 2 min. Exchange assays were stopped by the addition of 1 mL of ice-cold wash buffer, followed by filtration of the reaction mixture through a nitrocellulose filter (HAWP 02500, Millipore) which rapidly binds the remaining eIF-2•[^{3}H]-GDP complex. Filters were washed with 15 mL of ice-cold wash buffer to remove any unbound [^{3}H]-GDP.

Polyribosome Gradients. Hemin-supplemented protein synthesis mixes (100 μL) were incubated for 20 min at 30 °C with and without the additions noted in the figure legends. Samples were diluted with 150 μL of ice-cold gradient buffer and layered over 15−40% sucrose density gradients, and polyribosomal profiles were analyzed as described previously by centrifugation of samples in a Sorval AH650 rotor for 1 h at 45 000 rpm (*30, 50*). Inhibition of elongation was assayed by determining the ability of agents to inhibit runoff of polyribosomes after the addition of ATA, an initiation inhibitor. Specifically, hemin-supplemented protein synthesis mixes were supplemented with 120 μM ATA and incubated for 1.5 min at 30 °C with or without the additions noted in the figure legends prior to analysis as described above.

Western Blot Analysis. Samples were prepared for SDS−PAGE, separated in 10% gels, and transferred to a PVDF membrane as previously described (*41, 51*). Hsp70 and DnaJ were detected with a 1:1000 dilution of N27 anti-Hsp70 mAb or anti-DnaJ antibody, respectively.

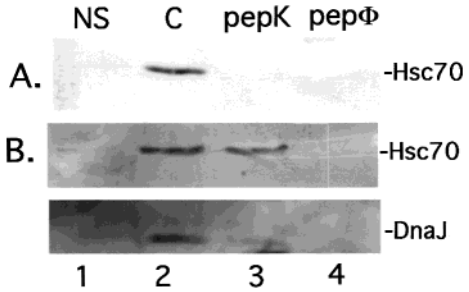

FIGURE 1: Effects of peptide-K or peptide-Φ on the interactions of chaperones with peptide-K-Sepharose and denatured luciferase. Peptide-K-Sepharose (A) or immobilized denatured luciferase (B) or control resins (NS) were incubated in RRL in the presence of DMSO (1% final concentration all samples as vehicle control) and no additions (lanes 1 and 2), 5 mM peptide-K (pepk, lane 3), or 0.5 mM peptide-Φ (pepΦ, lane 4) as described under Materials and Methods. After washing of resins, samples were analyzed by SDS−PAGE and western blotting with N27 anti-Hsc70 and anti-DnaJ antibodies as indicated in the figure.

Peptide Synthesis. Peptide-K (NIVRKKK) and peptide-Φ (FYQLALT) were synthesized by the Sarkey's Biotechnology Research Laboratory (OSU). The peptides were purified by C-18 reverse-phase chromatography at the Molecular Biology Resource Facility (University of Oklahoma Health Sciences Center, UOHSC), and the purity and concentrations of solutions of the peptides were verified by MALDI-mass spectroscopy and quantitative amino acid analysis using β-(thienyl)-DL-alanine as an internal standard. The analysis indicated that the peptides were of the expected mass and amino acid composition (i.e., >98% pure).

RESULTS

Peptide-K Specifically Binds Hsc70 in RRL. To determine the capacity of Hsc70 to specifically interact with peptide-K in RRL, peptide-K containing a Gly-Gly-Gly-Cys spacer on its C-terminus was cross-linked to agarose (peptide-K-agarose), and the ability of soluble peptide-K to compete specifically for the binding of proteins to the peptide-K-agarose was determined. Peptide-K-agarose was incubated in RRL, washed, and eluted by boiling in SDS−PAGE sample buffer. Western blot analysis indicated that Hsc70 bound peptide-K-agarose (Figure 1A, lane 2). Addition of soluble peptide-K quantitatively blocked the binding of Hsc70 to peptide-K-agarose (Figure 1A, lane 3), indicating that the interaction of Hsc70 with the peptide-K-agarose was saturable.

The ability of soluble peptide-Φ to compete for the binding of proteins to the peptide-K-agarose was also examined, as peptide-Φ inhibits the binding of purified Hsc70 to peptide-K in vitro (*26*). Consistent with this previous report, addition of peptide-Φ to RRL blocked the binding of Hsc70 to peptide-K-agarose (Figure 1A, lane 4). These results suggest that Hsc70 interacts with peptide-K in a saturable manner, and peptide-Φ interacts with Hsc70 in RRL in a manner similar to its interaction with purified Hsc70 in vitro.

Effect of Peptide-K and Peptide-Φ on Luciferase Renaturation in RRL. Renaturation of luciferase in RRL is facilitated by the Hsp90 chaperone machinery, of which Hsc70 is a component (*31*, *33*). In addition, luciferase renaturation can occur through an alternate kinetically slower Hsc70-dependent pathway, when the Hsp90-facilitated pathway is inhibited by the Hsp90-specific binding drug geldanamycin (*33*) or by the addition of molybdate (*34*). Luciferase renaturation can also be inhibited by agents that interact with Hsc70 directly, such as the Hsc70 antagonist clofibric acid (*32*, *52*, *53*) or denatured proteins [e.g., reduced−carboxymethylated bovine serum albumin, RCM-BSA, (*31*)]. Similarly, heat shock generates sufficient quantities of denatured protein in RRL to sequester the functional pool of Hsc70 in RRL and to inhibit the renaturation of luciferase (*31*, *42*).

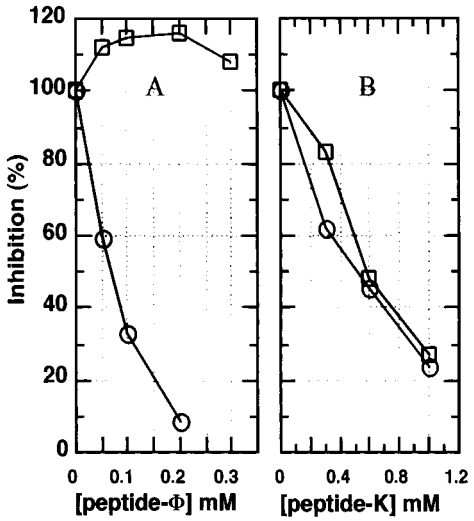

FIGURE 2: Effects of peptide-Φ (A) and peptide-K (B) on luciferase renaturation and protein synthesis in RRL. Denatured luciferase was incubated for 10 min in RRL in the presence of DMSO (1% final concentration all samples as vehicle control) and 0, 0.05, 0.1, and 0.2 mM peptide-Φ (A), or in water (vehicle control) and 0, 0.3, 0.6, and 1 mM peptide-K (B). Percent inhibition of luciferase renaturation (open circles) is expressed relative to the luciferase light production of the vehicle control sample in the absence of peptide. Hemin-supplemented protein synthesis mixes were incubated for 30 min in RRL in the presence of DMSO (1% final concentration vehicle control, 0 and 0.3 mM peptide-Φ) and 0, 0.05, 0.1, 0.2, and 0.3 mM peptide-Φ (A), or in water (vehicle control) and 0, 0.3, 0.6, and 1 mM peptide-K (B), and the amount of [^{35}S]-methionine incorporated into acid-precipitable protein was measured as described under Materials and Methods. Percent inhibition of protein synthesis (open squares) is expressed relative to the amount of [^{35}S]-methionine incorporated in the vehicle control sample in the absence of peptide.

The effect of peptide-K and peptide-Φ on the renaturation of thermally denatured luciferase in RRL was examined to determine whether these previously characterized Hsc70-binding peptides interfered with this Hsc70-dependent process. Both peptide-K and peptide-Φ inhibited the rate of luciferase renaturation in RRL in a concentration-dependent manner (Figure 2). The IC_{50} (concentration of peptide required to inhibit luciferase renaturation by 50%) for inhibition of luciferase renaturation by peptide-Φ and peptide-K was approximately 0.07 and 0.6 mM, respectively.

The effects of peptide-K and peptide-Φ on the binding of Hsc70 to denatured luciferase were examined to determine whether these peptides inhibited luciferase renaturation by blocking the binding of Hsc70 to denatured luciferase. Luciferase was adsorbed to anti-luciferase antibodies prebound to anti-IgG agarose, denatured, and incubated in RRL in the presence and absence of peptides. Samples were washed and analyzed for coadsorbed Hsc70 by SDS−PAGE and western blotting. Hsc70 was specifically coadsorbed with denatured luciferase from RRL (Figure 1B, upper panel).

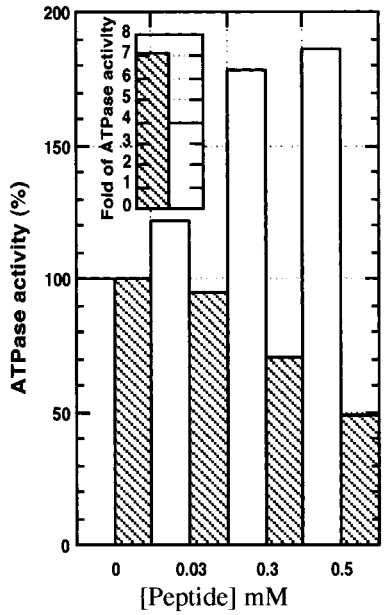

FIGURE 3: Effects of peptide-Φ and peptide-K on HDJ2-induced stimulation of Hsc70 ATPase activity. Hsc70 ATPase assays were performed as described under Materials and Methods. HDJ2 (0.25 μg/μL) and Hsc70 (1 μg/μL) were used for each assay. Peptide-Φ (open bars) and peptide-K (slashed bars) were used at the indicated concentrations (mM), and DMSO and H_2O were used as vehicle controls (0 mM peptide), respectively. Radioactivity was measured as phosphorimager disintegrations (PD) from [α-^{32}P]-ADP formed during the assay. The data are presented as percent of the HDJ2-stimulated Hsc70 ATPase activity in the presence of the vehicle controls, and the inset plot shows the fold stimulation of Hsc70 ATPase upon the addition of HDJ2 in the presence of the vehicle controls (H_2O, slashed bar; or DMSO, open bar). The average basal and HDJ2-stimulated Hsc70 ATPase activity after subtraction of the background for two experiments was 745 and 2970 PD in the presence of DMSO, and 698 and 4792 PD in the presence of water.

However, Hsc70 was not coadsorbed with the denatured luciferase in the presence of peptide-Φ. In contrast, addition of peptide-K had no apparent effect on the binding of Hsc70 to denatured luciferase. The inability of peptide-K to block the binding of Hsc70 to denatured luciferase suggested that if peptide-K was interacting with Hsc70, it was interacting with a site distinct from the heptameric hydrophobic peptide binding site on Hsc70 to which peptide-Φ is likely bound (i.e., the mode of interaction of Hsc70 with peptide-K is distinct from its mode of interaction with peptide-Φ).

Effect of Peptide-K and Peptide-Φ on HDJ2-Induced Stimulation of the ATPase Activity of Hsc70. Peptide-Φ and peptide-K have previously been shown to have different effects on Hsc70's ATPase activity. Peptide-Φ stimulated Hsc70's ATPase activity, while peptide-K had little or no effect (*26*). Sequence similarity between peptide-K and sequences in the DnaJ domain of HDJ2 led Hightower and co-workers to speculate that peptide-K may bind to the site within Hsc70 that interacts with DnaJ homologues rather than Hsc70's peptide-binding site (*26*).

To test this hypothesis, we examined the effect of peptide-Φ and peptide-K on the ability of HDJ2 to stimulate the ATPase activity of Hsc70 (Figure 3). Addition of HDJ2 stimulated the basal ATPase activity of Hsc70 approximately 7- and 4-fold over the water and DMSO vehicle controls, respectively (Figure 3, inset). The ATPase activity of Hsc70 was stimulated further upon addition of peptide-Φ in the presence of HDJ2. In contrast, peptide-K was observed to inhibit the ability of HDJ2 to stimulate Hsc70's ATPase activity in a concentration-dependent manner, with 0.5 mM peptide-K inhibiting Hsc70's HDJ2-stimulated ATPase activity by 50%.

The capacity of peptide-K to inhibit the ability of HDJ2 to stimulate the ATPase activity of Hsc70 suggested that it may be binding to a DnaJ interaction site on Hsc70. Therefore, we examined the effect of peptide-Φ and peptide-K on the interaction of DnaJ homologues with denatured luciferase (Figure 1B, lower panel). Western blotting with anti-DnaJ antiserum detected a protein band that was coadsorbed with denatured luciferase from control incubations in RRL (Figure 1B, lower panel, lane 2). However, relative to the control, only 8% of the amount of this protein was coadsorbed with denatured luciferase in the presence of peptide-K (Figure 1B, lower panel, lane 3), and none of this protein was detected coadsorbing with denatured luciferase in the presence of peptide-Φ (Figure 1B, lower panel, lane 4). This protein band was tentatively identified as the rabbit equivalent of HDJ2, as the band comigrated with purified HDJ2 on SDS−PAGE (not shown). HDJ2 from RRL was also detected bound to peptide-K-agarose when resin was washed with buffers containing low salt. However, no HDJ2 was detected when the resin was washed with buffer containing 150 mM NaCl (not shown), indicating that the binding of HDJ2 to peptide-K-agarose was not direct, but was mediated through a weak ionic interaction. Thus, the data suggest that peptide-K may inhibit luciferase renaturation by blocking the interaction of HDJ2 with luciferase-bound Hsc70.

Effect of Peptide-Φ and Peptide-K on Hsp90-Facilitated Kinase Maturation. Hsc70 is required for the assembly of the Hsp90 superchaperone complex with target client proteins [reviewed in (*54*, *55*)]. Recently, we have demonstrated that Hsc70 is required for Hsp90-dependent posttranslational folding of newly synthesized HRI into a conformation that is competent to undergo activation in response to heme-deficiency (*36*). While the association of the chaperones Hsc70 (*36*), Hsp90 (*35*), and p50^{cdc37} (*56*) with HRI occurs cotranslationally, we have been unable detect a cotranslational interaction of Hsp90 or p50^{cdc37} with the Hsp90-dependent Src-family tyrosine kinase, p56^{Lck} (B. Scroggins, S. Uma, S. D. Hartson, and R. L. Matts, manuscript in preparation). Therefore, we compared the effects of peptide-Φ and peptide-K on the maturation and activation of these two Hsp90-dependent protein kinases.

Peptide-Φ was found to inhibit the maturation and activation of HRI. Addition of peptide-Φ to heme-deficient RRL blocked the maturation and activation of HRI, which was apparent from the marked suppression of the autokinase and eIF2α kinase activity of HRI (Figure 4A, lanes 4 vs 6). Control incubations of HRI with peptide-Φ in vitro indicated that peptide-Φ had no direct effect on the kinase activity of HRI (not shown). Transformation of HRI into an active, heme-regulatable kinase occurs concomitant with a diagnostic electrophoretic mobility shift on SDS−PAGE (*35*, *36*). Upon maturation of HRI in heme-deficient RRL, 50% of the ^{35}S-labeled His-tagged HRI exhibited a slower electrophoretic mobility on SDS−PAGE, which is indicative of HRI transformation (Figure 4B, UF, lane 4). The ability of peptide-Φ to inhibit the transformation of newly synthesized

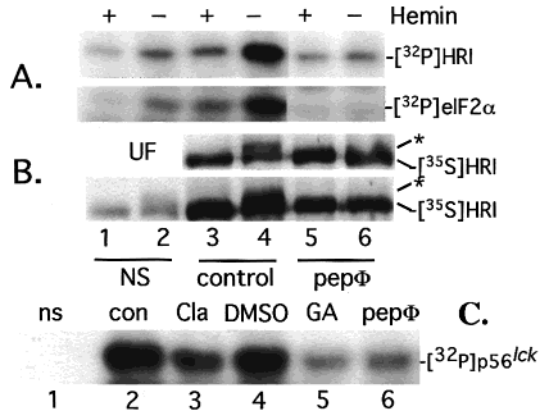

FIGURE 4: Effect of peptide-Φ on the maturation and activation of HRI in RRL. Newly synthesized [^{35}S]-(His_7)HRI (lanes 3−6) was generated de novo in RRL and matured in hemin-supplemented (+: lanes 1, 3, and 5) or heme-deficient RRL (−: lanes 2, 4, and 6) in the presence of 2% DMSO (vehicle control, lanes 1−4) or 0.5 mM peptide-Φ (lanes 5 and 6) for 1 h as described under Materials and Methods. HRI was affinity-purified by immunoadsorption with anti-His-tag antibodies and assayed for autokinase activity (A, upper panel) and eIF2α kinase activity (A, lower panel). [^{35}S]-HRI lacking the His-tag was synthesized and matured in RRL as the control for nonspecific binding to the affinity resin (NS, lanes 1 and 2). [^{35}S]-HRI present in an aliquot of unfractionated RRL (B: UF, lanes 3−6) and ^{35}S-labeled HRI immunoadsorbed from each of the RRL reaction mixes (B: lower panel) were detected by autoradiography. *, transformed HRI exhibiting slower electrophoretic mobility. (C) [^{35}S]-(His_7)p56^{Lck} (lanes 2−6) and nontagged [^{35}S]-p56^{Lck} (ns, lane 1) were generated de novo in RRL for 10 min followed by the addition of ATA. After polyribosome runoff (4 min), the p56^{lck} was matured in the presence of buffer (con, lane 2), 15 mM clofibric acid (Cla, lane 3), 1% DMSO (lane 4), 50 μg/mL geldanamycin (GA, lane 5), or 0.5 mM peptide-Φ (pepΦ, lane 6) for 15 min followed by immunoadsorption with anti-His-tag antibodies. Samples were then assayed for autokinase activity [^{32}P]-p56^{Lck} as described previously. Autoradiography of [^{35}S]-(His_7)p56^{Lck} showed that equivalent amounts of p56^{Lck} were immunoadsorbed from RRL (not shown).

HRI is supported by the following observations: (1) little or no [^{35}S]-HRI exhibited retarded electrophoretic mobility when incubated in heme-deficient RRL in the presence of peptide-Φ; and (2) little or no [^{35}S]-HRI exhibiting retarded electrophoretic mobility was immunoadsorbed from peptide-Φ-treated RRL (Figure 4B, lanes 4 vs 6).

To determine whether the effect of peptide-Φ on kinase maturation was specific to polypeptides (e.g., HRI) that associate with Hsp90 and p50^{cdc37} cotranslationally, we examined the effect of peptide-Φ on the maturation and activity of newly synthesized p56^{Lck} kinase. Peptide-Φ inhibited the maturation of p56^{Lck} into an active kinase to the same degree that the maturation of p56^{Lck} was blocked by the Hsp90 inhibitor geldanamycin (Figure 4C). The Hsc70 inhibitor clofibric acid also inhibited the activation of p56^{Lck}. However, none of these agents had any direct effect on the autokinase activity of mature p56^{Lck} in kinase assays carried out in vitro (not shown). Thus, like HRI (*36*), the Hsp90-dependent maturation of p56^{Lck} required Hsc70.

In contrast to the effect of peptide-Φ, peptide-K caused a stimulation rather than an inhibition of HRI activity. Quantification of band intensity indicated that addition of 5 mM peptide-K during the maturation of newly synthesized HRI in heme-deficient RRL stimulated the autokinase and eIF2α kinase activities of HRI by approximately 2-fold and 4-fold (Figure 5, upper and middle panels), respectively. In hemin-supplemented RRL, addition of 5 mM peptide-K caused an approximate 8-fold and 9-fold stimulation of HRI's autokinase and eIF2α kinase activities, respectively. Consistent with its lack of any inhibitory effect on HRI activity, peptide-K had little effect on the amount of [^{35}S]-HRI exhibiting retarded electrophoretic mobility which was immunoadsorbed from heme-deficient RRL (Figure 5, lower panel). Quantification of the [^{35}S]-HRI bands indicated that equivalent amounts of HRI were adsorbed from the RRL reaction mixtures. Thus, peptide-Φ and peptide-K were observed to have different effects on the maturation and activation of HRI.

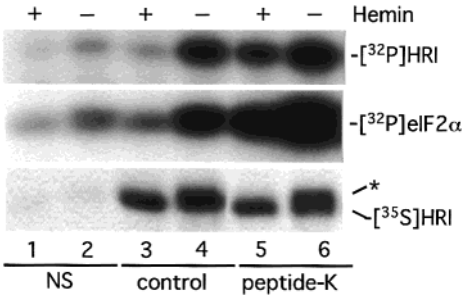

FIGURE 5: Effect of peptide-K on the maturation and activation of HRI in RRL. Newly synthesized [^{35}S]-(His_7)HRI (lanes 3−6) was generated de novo in RRL and matured in hemin-supplemented (+: lanes 1, 3, and 5) or heme-deficient RRL (−: lanes 2, 4, and 6) supplemented with water (vehicle control, lanes 1−4) or 5 mM peptide-K (lanes 5 and 6) for 1 h as described under Materials and Methods. HRI was affinity-purified by immunoadsorption with anti-His-tag antibodies and assayed for autokinase activity (upper panel) and eIF2α kinase activity (middle panel). ^{35}S-labeled HRI immunoadsorbed from each RRL reaction mix were detected by autoradiography (lower panel). *, transformed HRI exhibiting slower electrophoretic mobility.

To determine whether peptide-Φ inhibited the interaction of Hsc70 with HRI during its maturation in RRL, we examine the effect of peptide-Φ on the ability of anti-Hsc70 antibody to coadsorb [^{35}S]-HRI. Peptide-Φ-induced inhibition of HRI transformation (Figure 6A, UF, lanes 4 vs 6) correlated with a marked reduction in the amount of newly synthesized and mature-competent HRI that was coimmunoadsorbed with Hsc70 from either heme-supplemented or heme-deficient RRL (Figure 6A, lower panel, lanes 3 and 4 vs 5 and 6). Consistent with its lack of effect on HRI transformation (Figure 5 and Figure 6B, UF), peptide-K had little or no effect on the amount of mature-competent HRI that the was coimmunoadsorbed with Hsc70 from RRL (Figure 6B). Thus, the ability of peptide-Φ to inhibit HRI maturation and activation correlated with its ability to suppress the interaction of Hsc70 with newly synthesized HRI.

Effects of Peptide-K and Peptide-Φ on Protein Synthesis in RRL. Hsc70 negatively attenuates the activation of transformed HRI in response to heme-deficiency, and heat or oxidative stress (*30*). We have hypothesized that agents which bind Hsc70, and thus deplete the pool of free Hsc70 available to interact with HRI, would induce the activation of HRI and cause protein synthesis to become inhibited (*30, 42, 51*). Hence, the effects of peptide-K and peptide-Φ on protein synthesis in RRL were examined (Figure 7A). Peptide-K inhibited protein synthesis in a concentration-dependent manner, with an IC_{50} for translational inhibition of 0.5 mM (Figure 2B). The IC_{50} for peptide-K-induced

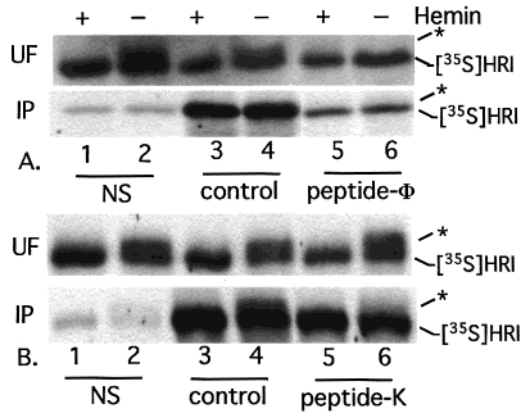

FIGURE 6: Effect of peptide-Φ and peptide-K on the interaction of Hsc70 with newly synthesized HRI in RRL. Newly synthesized [^{35}S]-HRI was generated de novo in RRL and matured in hemin-supplemented (+: lanes 1, 3, and 5) or heme-deficient RRL (−: lanes 2, 4, and 6) supplemented with (A) 2% DMSO (vehicle control, lanes 1−4) or 0.5 mM peptide-Φ (lanes 5 and 6), or with (B) water (vehicle control, lanes 1−4) or 5 mM peptide-K (lanes 5 and 6) for 1 h as described under Materials and Methods. Hsc70 was immunoadsorbed with BB70 anti-Hsc70 monoclonal antibodies (IP: lanes 3−6) or nonimmune control monoclonal antibodies (IP: lanes 1 and 2). ^{35}S-labeled HRI immunoadsorbed from each RRL reaction mix (A and B: IP, lower panels) was detected by autoradiography. [^{35}S]-HRI lacking the His-tag was synthesized and matured in RRL as the control for nonspecific binding to the affinity resin (NS, lanes 1 and 2). *, transformed HRI exhibiting slower electrophoretic mobility.

translational inhibition correlated quite well with the 0.6 mM IC_{50} for peptide-K-induced inhibition of luciferase renaturation. Unlike the biphasic inhibition of translation induced by RCM-BSA (*42*), peptide-K inhibited the translation rate immediately upon its addition to RRL, with the translation rate gradually declining at longer incubation times (Figure 7A). These kinetics of inhibition suggested that peptide-K was inhibiting translation at the elongation stage. Surprisingly, peptide-Φ was found to have no effect on protein synthesis in RRL (Figure 7A), even when added at levels that inhibited the renaturation of luciferase by greater than 90% (Figure 2A).

To characterize the mechanism of translational inhibition induced by peptide-K, polyribosome profiles of peptide-treated RRL translations were analyzed. Polyribosome profiles indicated that peptide-K induced the accumulation of 80S ribosomes (Figure 7B, ii), indicating inhibition of translation at the level of initiation. However, while the amount of polyribosomes present in peptide-K-treated RRL was reduced, polyribosomes were still present in the profiles, suggesting again that peptide-K also inhibited translation at the level of elongation. To determine whether peptide-K inhibits elongation, polyribosome runoff experiments were performed. The polyribosomes present in control RRL ran off within 1.5 min after the addition of the initiation inhibitor ATA, with the accompanying accumulation of 80S ribosomes (Figure 7B, iii). However, polyribosomes were still present in peptide-K-treated RRL after 1.5 min of incubation in the presence of ATA, indicating that peptide-K inhibited the rate of elongation in RRL (Figure 7B, iv). In contrast to peptide-K, peptide-Φ had no effect on the polyribosome profiles or on polyribosome runoff in RRL (not shown), consistent with the lack of any inhibitory effect of peptide-Φ on translation.

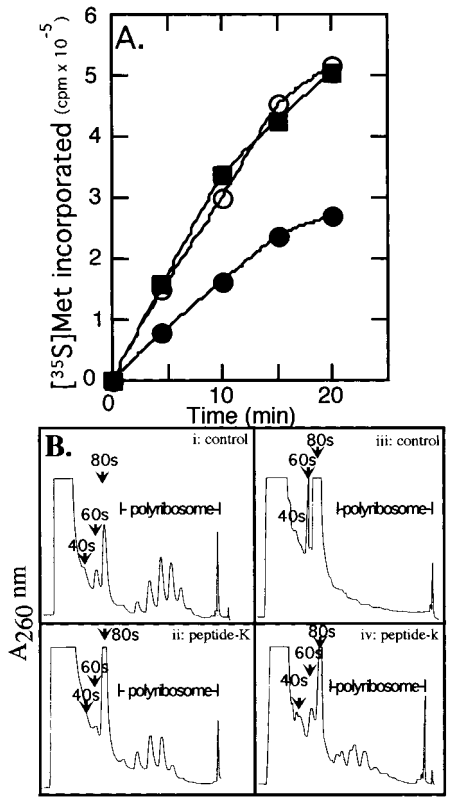

FIGURE 7: Effect of peptide-Φ and peptide-K on protein synthesis in hemin-supplemented RRL. RRL was incubated under conditions for protein synthesis at 30 °C in the presence of 10 μM hemin and 1% DMSO without (open circles) or with the addition of 0.3 mM peptide-Φ (closed squares) or 0.5 mM peptide-K (closed circles). (A) Protein synthesis was measured by the incorporation of [^{35}S]-methionine into acid-precipitable protein at the times indicated in the figure. (B) Polyribosome profiles of hemin-supplemented protein synthesis mixes incubated for 20 min at 30 °C in the absence (i and iii) or presence (ii and iv) of 5 mM peptide K without (i and ii) or with (iii and iv) the addition of the initiation inhibitor ATA 1.5 min prior to the analysis. Polyribosome profiles were analyzed as described as described under Materials and Methods.

Effects of Peptide-K and Peptide-Φ on the Activation of HRI in RRL. The increase in amount of 80S ribosomes present in RRL supplemented with peptide-K suggested that initiation of protein synthesis was also inhibited in the presence of peptide-K. Therefore, we determined the effect of peptide-K and peptide-Φ on the phosphorylation status of eIF-2α in RRL. Western blotting of isoelectric focusing gels indicated that addition of peptide-K to RRL induced an increase in the amount of phosphorylation of eIF-2α (Figure 8A). In contrast, addition of peptide-Φ to RRL had no effect on eIF-2α phosphorylation.

The effect of peptide-K on eIF-2B-catalyzed guanine nucleotide exchange from eIF-2·[^{3}H]-GDP complexes in RRL was determined. The guanine nucleotide exchange activity of eIF-2B was inhibited by 70% in RRL supplemented with peptide-K (Figure 8B). Consistent with the lack of an effect of peptide-Φ on protein synthesis and eIF-2α phosphorylation, addition of peptide-Φ had no effect on eIF-2B activity in RRL. Thus, peptide-K induced phosphorylation of eIF-2α to a level that was sufficient to significantly inhibit eIF-2B activity and account for peptide-K-induced inhibition of the initiation of protein synthesis in RRL.

To determine whether peptide-K was causing the activation of HRI, a homogeneous population of ^{35}S-labeled His-tagged

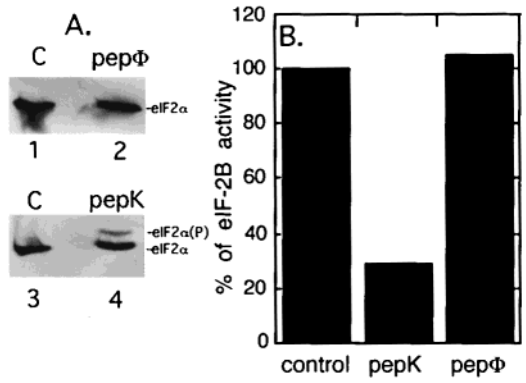

FIGURE 8: Effect of peptide-Φ and peptide-K on eIF2α phosphorylation and eIF2B activity in hemin-supplemented RRL. RRL was incubated under conditions for protein synthesis at 30 °C in the presence of 10 μM hemin and 1% DMSO without (lanes 1 and 3) or with the addition of 0.3 mM peptide-Φ (lane 2) or 0.5 mM peptide-K (lane 4). (A) eIF2α phosphorylation levels were determined by VSIEF of 5 μL aliquots of RRL that were removed for analysis after 15 min of incubation, followed by western blotting with anti-eIF2α antibodies. (B) The guanine nucleotide exchange activity of eIF2B was measured after 20 min incubation of the hemin-supplemented RRL containing 1% DMSO (control, 1), 5 mM peptide-K (pepK), or 0.3 mM peptide-Φ (pepΦ). Values are expressed as percent of eIF2-bound [^{3}H]-GDP exchanged in 2 min relative to the control.

HRI was synthesized de novo and then: (i) matured in hemin-supplemented RRL to yield mature-competent HRI; (ii) matured in heme-deficient RRL to yield transformed HRI; or (iii) matured and transformed in heme-deficient RRL followed by the addition of hemin to yield repressed HRI [for a detailed description of these defined forms of HRI, see refs (*35*, *36*)]. The RRLs containing the mature-competent, transformed, or repressed His-tagged [^{35}S]-HRI were then incubated with peptide-K, peptide-Φ, or DMSO as the vehicle control. The His-tagged [^{35}S]-HRI was then immunoadsorbed with anti-His-tag antibodies, and HRI's autokinase and eIF2α kinase activities were assessed. The autokinase and eIF2α kinase activities of HRI adsorbed from peptide-K-treated heme-deficient or hemin-supplemented RRL (Figure 9, lanes 6−8) were enhanced compared to control RRL (Figure 9, lanes 3−5). In contrast, addition of peptide-Φ (Figure 9, lanes 9−11) appeared to have a slight inhibitory effect on HRI's autokinase and eIF2α kinase activities compared to the controls.

Effects of Peptide-K and Peptide-Φ on the Interaction of Hsc70 with Endogenous HRI. We have previously hypothesized that agents which disrupt the interaction of Hsc70 with mature HRI will activate HRI by suppressing the ability of Hsc70 to attenuate HRI activation (*30*, *36*, *42*). Thus, the activation of HRI induced by peptide-K in RRL would be predicted to be accompanied by the inhibition of the interaction of Hsc70 with HRI. Furthermore, it predicts that peptide-Φ should have no effect on the interaction of Hsc70 with HRI, as peptide-Φ does not induce HRI activation. Therefore, we immunoprecipitated HRI from control RRL and RRL treated with either peptide-K or peptide-Φ. Control incubations were carried out in the presence of BSA and RCM-BSA. Consistent with our previous work (*36*, *42*), addition of RCM-BSA blocked the interaction of Hsc70 with HRI, while BSA had no effect on this interaction (Figure 10, lanes 6 versus 5). Similar to the effect of RCM-BSA, peptide-K blocked the interaction of Hsc70 (Figure 10, lane 3), while peptide-Φ had no effect (Figure 10, lane 4). Thus, the capacity of peptide-K to cause the activation of HRI in RRL correlated with its ability to block the interaction of Hsc70 with endogenous HRI.

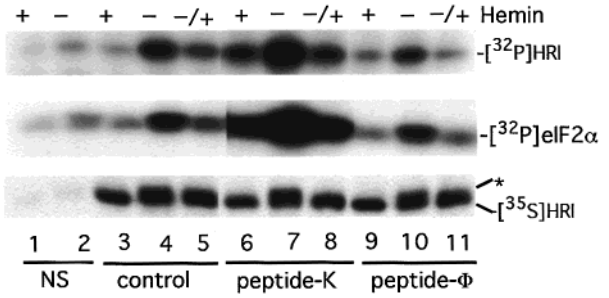

FIGURE 9: Effect of peptide-Φ and peptide-K on the activity of matured HRI in RRL. Mature-competent (+), transformed (−), and repressed (−/+) [^{35}S]-(His$_7$)HRI (lanes 3−11) were generated de novo in RRL as described under Materials and Methods. Mature-competent and transformed non-His-tagged [^{35}S]-HRI were generated as controls for nonspecific binding of activity from RRL (NS, lanes 1 and 2). Samples were then supplemented with 1% DMSO (lanes 1−11) and water (NS and control: lanes 1and 2, and 3−5), and 5 mM peptide-K (lanes 6−8) or 0.5 mM peptide-Φ (lanes 9−11) and incubated for another 20 min. HRI was affinity-purified by immunoadsorption with anti-His-tag antibodies and assayed for autokinase ([^{32}P]HRI: upper panel) and eIF2α kinase ([^{32}P]eIF2α: middle panel) activity. [^{35}S]-HRI present in immunoprecipitates ([^{35}S]HRI: lower panel) was detected by direct autoradiography. *, transformed HRI.

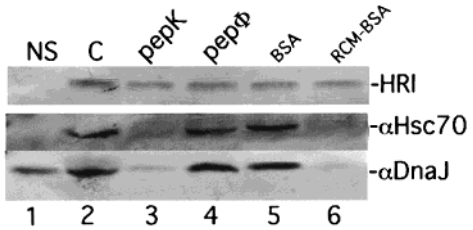

FIGURE 10: Effect of peptide-Φ and peptide-K on the interaction of Hsc70 and HDJ2 with endogenous HRI in RRL. Hemin-supplemented RRLs were incubated under protein synthesis conditions at 30 °C for 20 min in the presence of 1% DMSO without (lanes 1 and 2) or with the addition of 5 mM peptide-K (lane 3), 0.5 mM peptide-Φ (lane 4), 1.5 mg/mL BSA (lane 5), or 1.5 mg/mL RCM-BSA (lane 6). HRI was immunoadsorbed to resin-bound nonimmune (NS: lane 1) or anti-HRI (lanes 2−6) IgY. Samples were analyzed by SDS−PAGE, and HRI (upper panel), Hsc70 (middle panel), and HDJ2 (lower panel) were detected by western blotting as described under Materials and Methods.

HDJ2 Potentiates the Ability of Hsc70 To Attenuate HRI Activation in RRL. The ability of peptide-K to cause the activation of HRI in RRL and its ability to inhibit HDJ2-induced stimulation of Hsc70's ATPase activity suggested that DnaJ homologues may play a role in Hsc70-modulated attenuation of HRI activation in RRL. To test this hypothesis, heme-deficient RRL was incubated in the presence or absence of HDJ1 or HDJ2 with or without the addition of Hsc70 (Figure 11A). Addition of either HDJ1 (not shown) or HDJ2 alone had no effect on the shut-off of protein synthesis in response to heme-deficiency. However, HDJ2 markedly potentiated the ability of Hsc70 to attenuate the shut-off of protein synthesis in heme-deficient RRL (Figure 11A), while HDJ1 had no effect (not shown). The ability of HDJ2 to potentiate Hsc70's ability to maintain protein synthesis in heme-deficient RRL correlated with decreased levels of eIF2α phosphorylation (Figure 11B, lane 4). These

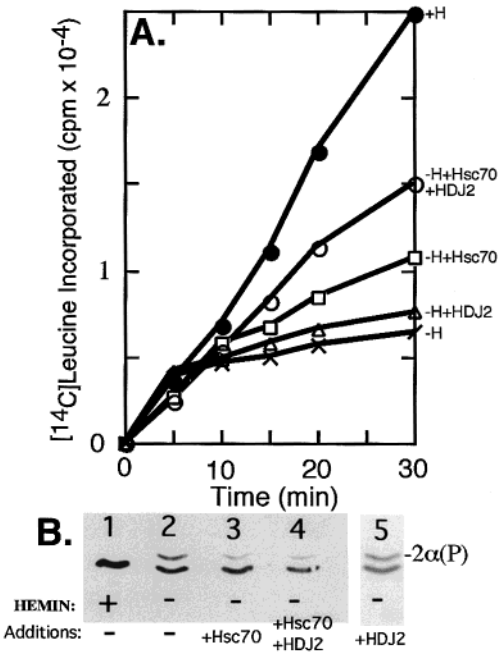

FIGURE 11: Effect of HDJ2 on protein synthesis and eIF2α phosphorylation in heme-deficient RRL. RRL was incubated under conditions for protein synthesis in the presence of 10 μM hemin (+H, closed circles) or in the absence of heme with the addition of buffer (−H, ×), 100 μg/mL Hsc70 (−H+Hsc70, open squares), 2.5 μg/mL HDJ2 (−H+HDJ2, open triangles), or 100 μg/mL Hsc70 and 2.5 μg/mL HDJ2 (−H+Hsc70+HDJ2, open circles) at 30 °C. (A) Protein synthesis was measured by the incorporation of [^{14}C]-leucine into acid-precipitable protein at times indicated in the figure. (B) eIF2α phosphorylation levels were determined by VSIEF of 5 μL aliquots of RRL that were removed for analysis after 5 min of incubation, followed by western blotting with anti-eIF2α antibodies.

results suggest that HDJ2, but not HDJ1, may play a role in modulating the interaction of Hsc70 with transformed HRI in RRL.

To determine whether HDJ2 might interact directly with HRI, blots of anti-HRI immunoresins were probed with anti-DnaJ antibody. The western blot indicated that a immunoreactive protein with the electrophoretic mobility of HDJ2 was coadsorbed with HRI from RRL (Figure 10, lower panel). However, upon the addition of peptide-K to RRL, little HDJ2 was coadsorbed with HRI (Figure 10, lane 3). On the other hand, peptide-Φ had no effect on the amount of HDJ2 that was coadsorbed with HRI. These observations suggest that HDJ2 plays a role in Hsc70-mediated attenuation of HRI activation, and further support the hypothesis that peptide-K does not interact with the peptide-binding pocket of Hsc70, but rather that it may interact with or near a site on Hsc70 to which DnaJ homologues may bind.

Effects of a SH3-Domain-Binding Peptide on Luciferase Renaturation and Protein Synthesis. The effects of a SH3-domain-binding peptide (peptide-SH3: SPPTPKPRPPRP-LPVAPGS) on a number of the experimental parameters investigated above were also examined. Peptide-SH3 blocked the binding of Hsc70 and HDJ2 to peptide-K-agarose (not shown). Luciferase renaturation was inhibited by peptide-SH3 with an IC_{50} of ∼2 mM, which correlated with the ability of peptide-SH3 to block the binding of HDJ2 to luciferase and decrease the binding of Hsc70 to luciferase from 75 to 95% (not shown). Peptide-SH3 also blocked the binding of Hsc70 and HDJ2 to HRI (not shown) and inhibited protein synthesis with a IC_{50} of ∼6 mM. Thus, peptide-SH3 had properties of both peptide-K and peptide-Φ, which is consistent with the peptide containing both a tract of hydrophobic amino acid residues and a number of positively charged amino acids.

DISCUSSION

Previous work had identified two peptide sequences to which Hsc70 binds with high affinity. These peptides had distinct effects on the in vitro ATPase activity of Hsc70 and bound to different conformations of purified Hsc70 (*26*), suggesting that the peptides might affect the function of Hsc70 differently. In the work presented here, we tested this hypothesis and demonstrated that peptide-K and peptide-Φ indeed have distinct effects on Hsc70-mediated processes in RRL.

Previous work suggested that peptide-Φ bound Hsc70 and stimulated its ATPase activity, causing Hsc70 to adopt its "closed" ADP-bound conformation with peptide-Φ occupying Hsc70's hydrophobic peptide-heptamer-binding cleft (*26*). Consistent with this notion, peptide-Φ-induced inhibition of luciferase renaturation in RRL correlated with the ability of peptide-Φ to block the binding of Hsc70 and HDJ2 to denatured luciferase. Furthermore, peptide-Φ increased HDJ2-induced stimulation of Hsc70's ATPase activity, a process that is known to be stimulated by DnaJ homologues upon the interaction of Hsc70 with a binding substrate (*4*, *6*, *7*). Thus, high-affinity binding of peptide-Φ to the peptide-binding cleft of Hsc70 appears to block the interaction of Hsc70 with denatured luciferase, thereby inhibiting luciferase renaturation.

On the other hand, previous work suggested that peptide-K interacted with Hsc70 in its "open" ATP-bound conformation (*26*). Since peptide-K did not stimulate Hsc70's ATPase activity, it was postulated either that peptide-K bound to the peptide-binding cleft in a manner distinct from peptide-Φ or that peptide-K specifically interacted with some other site on Hsc70. Sequence similarities between peptide-K and sequences within the J-domain of HDJ2 lead to the hypothesis that peptide-K and HDJ2 may interact with a common site. Consistent with this latter hypothesis, we observe that: (i) peptide-K specifically interacted with Hsc70 and inhibited luciferase renaturation in RRL; (ii) peptide-K blocked the binding of HDJ2 to denatured luciferase, but had no effect on the interaction of Hsc70 with luciferase; and (iii) peptide-K inhibited the ability of HDJ2 to stimulate Hsc70's ATPase activity.

Thus, peptide-K appears to interfere with the ability of DnaJ homologues (e.g., HDJ2) to interact with the Hsc70. The ability of peptide-K to inhibit luciferase renaturation, without blocking the interaction of Hsc70 with denatured luciferase, suggests that peptide-K does not compete directly for the peptide-binding site on Hsc70. Rather it implies that peptide-K must interact outside the peptide-binding pocket of Hsc70, binding at a site critical for the regulation of Hsc70 function. Previous work has demonstrated that HDJ1 (*57*) and HDJ2 (*58*) stimulate Hsc70-facilitated renaturation of luciferase. The observation that peptide-K decreased the amount of HDJ2 that coadsorbed with denatured luciferase (Figure 1B) is consistent with the notion that peptide-K inhibits Hsc70-facilitated luciferase renaturation by inhibiting

the interaction of DnaJ homologues with Hsc70, resulting in the inhibition of Hsc70's ATP-dependent reaction cycle. This hypothesis is supported by biochemical and genetic data that indicate the binding of the J-domain of DnaJ to the bacterial Hsp70 homologue DnaK involves the interaction of conserved positively charged residues in the J-domain with conserved acidic residues present within a cleft of the ATPase domain of DnaK (*59*−*61*).

The distinct pharmacological activities of the two Hsc70-binding peptides were further illustrated by their contrasting effects on the maturation of newly synthesized kinases. Peptide-Φ inhibited the Hsp90/Hsc70-dependent maturation and activation (*35*, *36*) of newly synthesized HRI in RRL. This inhibition correlated with the ability of peptide-Φ to inhibit the interaction of Hsc70 with newly synthesized untransformed HRI. The inhibitory effect of peptide-Φ on kinase maturation was not specific to HRI, to which Hsc70 and Hsp90 associate cotranslationally (*35*, *36*), as peptide-Φ also inhibited the maturation and activation of $p56^{Lck}$, to which Hsp90 appears to bind after its release from the ribosome (B. Scroggins, S. Uma, S. Hartson, and R. L. Matts, manuscript in preparation). At the current time, we cannot rule out the possibility that peptide-Φ may have additional effects on Hsp90 activity, which is also required for the maturation and activation of these kinases. In contrast to peptide-Φ, peptide-K had no inhibitory effect on the maturation and activation of HRI.

Peptide-Φ had effects on protein folding and renaturation similar to those of the Hsp90-specific inhibitor geldanamycin. Peptide-Φ was found to have no effect on the synthesis, folding, and activity of newly synthesized luciferase (B.-G. Yun and R. L. Matts, unpublished observations). While Hsp90 is not absolutely required for the renaturation of luciferase in vitro (*58*), in RRL the kinetically preferred pathway for luciferase renaturation is catalyzed by the Hsp90 chaperone complex, of which Hsc70 is a component (*33*). Similar to the effects of peptide-Φ, the Hsp90-binding drug geldanamycin inhibits luciferase renaturation (*33*, *58*), but it has no effect on the synthesis, folding, or activity of newly synthesized luciferase (*62*, *63*). Therefore, we speculate that peptide-Φ may act, in part, through inhibition of Hsc70-dependent functions within the Hsp90 superchaperone complex.

In contrast to peptide-Φ, peptide-K inhibited protein synthesis at the level of both initiation and elongation. Cytosolic Hsp70 associates with nascent polypeptide chains cotranslationally (*64*−*68*), and has been suggested to play a role in elongation by aiding the passage of nascent polypeptide chains through the ribosomal pore (*69*). On the other hand, there is currently no firm consensus on whether DnaJ homologues stably bind nascent polypeptides in vivo (*10*, *65*). While Hsp40 (HDJ1) has been reported to associate with luciferase cotranslationally in RRL (*65*), nascent/newly synthesized proteins have not been observed in chaperone complexes with either HDJ1 or HDJ2 in HeLa cells (*10*). However, there is general agreement that DnaJ homologues function to regulate the Hsp70 ATPase cycle and the stability of Hsp70−substrate complexes (*4*, *7*−*10*). Thus, it is tempting to speculate that peptide-K-induced inhibition of elongation may be the result of its ability to block the interaction of a DnaJ homologue(s) with ribosome-bound Hsp70, arresting Hsp70's ATPase cycle and inhibiting Hsp70-dependent "ratcheting" of nascent polypeptide chains on the ribosome. However, other mechanisms are conceivable, as the positively charged peptide-K could interact with the phosphodiester backbone of RNAs and disrupt any number of protein−RNA interactions required for translation to occur normally.

Peptide-K also induced an inhibition of polypeptide chain initiation that was evident by the accumulation of inactive 80S ribosomes in the polyribosome profiles of heme-supplemented RRL. This inhibition of initiation correlated with the ability of peptide-K to block the interaction of Hsc70 and HDJ2 with HRI, and was associated with the activation of HRI and an increase in the level of eIF2α phosphorylation that was sufficient to inhibit eIF-2B activity. The absence of an effect of peptide-Φ on HRI activation indicates that Hsc70 does not simply bind to transformed HRI directly through the recognition of some feature of HRI that mimics an exposed heptameric sequence of hydrophobic amino acids, such as might direct the interaction of Hsc70 with denatured substrates.

The data suggest that a DnaJ homologue, likely HDJ2, modulates the interaction of Hsc70 with transformed HRI. In addition to their J-domains, which modulate the interaction of DnaJ homologues with the ATPase domain of Hsp70 family members, DnaJ homologues contain unique domains that direct their interaction with other proteins (*4*−*6*, *18*, *19*, *70*). The ability of HDJ2, but not HDJ1, to potentiate the capacity of exogenous Hsc70 to suppress HRI activation indicates that there is specificity in the mechanism by which the binding of Hsc70 to transformed HRI is modulated. HDJ2 contains a Cys-rich polypeptide-binding motif that is lacking in HDJ1 (*70*−*72*), which may modulate its interaction with HRI.

Overall, these results indicate that Hsc70 binds differently to folding or denatured proteins in comparison to its regulatory interactions with mature proteins. Furthermore, these interactions appear to have different dependencies on DnaJ homologues. Currently, little is known about how Hsc70 binds to "native" substrates and whether its regulatory interaction requires the conventional heptameric substrate-binding site. However, it should be emphasized that the two sites with which the peptides interact cannot technically be considered to be "independent", as binding of peptide-Φ to Hsc70's heptameric peptide-binding site precludes the interaction of Hsc70 with peptide-K.

Hsc70 and related family members play roles in modulating processes critical for the regulation of all aspects of a cell's physiology, making it an attractive target for pharmacological intervention. The Hsc70-binding drug 15-deoxyspergualin is currently being investigated as a possible immunosuppressant (*73*, *74*). In addition, treatment of cells expressing a mutant form of the cystic fibrosis transmembrane conductance regulator (ΔF508-CFTR) with 15-deoxyspergualin was shown to enhance the function of this mutant protein (*75*), probably by inducing the dissociation of Hsc70 from ΔF508-CFTR and freeing it from sequestration in the endoplasmic reticulum. 15-Deoxyspergualin stimulates the ATPase activity of Hsc70, but does not inhibit DnaJ-induced stimulation of Hsc70 ATPase activity or the binding and release of Hsc70 to peptide substrates (*76*). Recently, the antimicrobial activities of the peptides pyrrhocoricin, drosocin, and apidaecin were found to correlate with their binding

affinity for DnaK (the bacterial Hsp70 homologue) and inhibition of its ATPase activity (*77*, *78*).

The findings presented in this report indicate that two previously identified high-affinity Hsc70-binding peptides (*26*) have distinct pharmacological properties, and differentially inhibit various physiological functions of Hsc70. Peptide-Φ-induced inhibition of Hsc70 function occurred in the absence of a generalized inhibitory effect on protein synthesis and protein folding, indicating specific Hsc70 functions can be targeted pharmacologically without a complete disruption of Hsc70's "house-keeping" functions in the cell. In addition, the data indicate that regulatory functions of Hsc70 can be inhibited (e.g., peptide-K-induced inhibition of Hsc70-dependent attenuation of HRI activation) in the absence of an effect on the biogenesis of functional proteins (e.g., Hsp90/Hsc70-dependent transformation of HRI). Furthermore, Hsc70's biogenic function could be inhibited (e.g., peptide-Φ-induced inhibition of HRI and $p56^{Lck}$ maturation) in the absence of an effect on a regulatory function of Hsc70 (e.g., attenuation of HRI activation). Thus, these results and those of others (*73*−*75*, *77*, *78*) indicate that it should be feasible to develop therapeutically valuable agents that target specific Hsp70-dependent processes within cells for pharmacological intervention in the treatment of disease.

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