Characterization of Functional Domains of the Eukaryotic Co-chaperone Hip*

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The homo-oligomeric Hip protein cooperates with the 70-kDa heat shock cognate Hsc70 in the folding of newly synthesized polypeptide chains and in the conformational regulation of signaling molecules known to interact with Hsc70 and Hsp90. In order to further assess the role of Hip during protein biogenesis, a structure-function analysis of the Hip protein was initiated. By employing the yeast two-hybrid system, the Hsc70-binding site of Hip was mapped to a domain comprising multiple tetratricopeptide repeats and flanking charged α-helices. Affinity chromatography confirmed direct interaction of isolated Hip fragments and protein fusions bearing this region with the ATPase domain of Hsc70 in an ATP- and salt-dependent manner. Contact of Hip with the ATPase domain appears to be mediated primarily by the positively charged α-helix following the tetratricopeptide repeats. Furthermore, a domain required for homo-oligomerization was identified at the extreme amino terminus of Hip.

Molecular chaperones of the 70-kDa Hsps1 play a key role in intracellular protein biogenesis due to their ability to stabilize nonnative protein conformations (1–3). Chaperone activity is achieved through a dynamic cycle of binding and release of the nonnative polypeptide substrate coupled to a cycle of ATP binding and hydrolysis by the chaperone protein. The ATP dependence of the reaction confers the means of extensive regulation through the function of cooperating proteins that influence the nucleotide state of Hsp70 and thus modulate its affinity for polypeptide substrate. In Escherichia coli, cycling of the Hsp70 homologue DnaK is regulated by the DnaJ protein, which promotes the formation of DnaK-substrate complexes by stimulating DnaK’s ATPase activity and by the nucleotide exchange factor GrpE required for complex dissociation (4–6). Although the bacterial reaction cycle served for a long time as a paradigm for the regulation of Hsp70 chaperone proteins, the recent identification of the Hsc70-interacting protein Hip reveals a distinct regulatory mechanism in the eukaryotic cytosol (7). Hip directly binds to the ATPase domain of Hsc70 dependent on the activation of the Hsc70 ATPase by the mammalian DnaJ homologue Hsp40 (8, 9). Interaction with Hip stabilizes the ADP-bound state of Hsc70 corresponding to the conformation of Hsc70 that most stably binds polypeptide substrate (10). Apparently, Hsc70 activity in the eukaryotic cytosol is regulated through a Hip-mediated stabilization of chaperone-substrate complexes, in contrast to the GrpE-mediated dissociation characteristic of the prokaryotic reaction cycle (11).

By stabilizing Hsc70-substrate complexes, Hip may provide the molecular basis for an efficient cooperation of Hsc70 with other chaperone systems in the eukaryotic cytosol. For example, Hsc70 cooperates with Hsp90 during the conformational regulation of certain proteins involved in signal transduction such as tyrosine kinases and steroid hormone receptors (12–15). Intriguingly, the human Hip homologue has recently been identified as the 48-kDa component, p48, of steroid receptor-chaperone complexes (16). By presenting the receptor in a conformation that can be recognized by Hsp90, Hip in concert with Hsc70 and a DnaJ homologue appear to mediate early stages of complex assembly (16–20). During the assembly process additional factors such as the Hsp90 partner protein p60, the homologue of yeast Sti1 (21), may promote further cooperation between the chaperone systems (17, 22–24). However, the association of Hip, Hsc70, the DnaJ homologue, and p60 with the receptor-Hsp90 complex is transient. The proteins are not present in the mature complex that confers high affinity hormone binding and that contains as additional components p23 and the immunophilins CyP-40 and FKBP52 (17, 24–26). Apparently, successive conformational changes of the signaling molecule require the concerted action of Hsc70 and Hsp90 as well as their respective co-chaperones.

In the light of their close cooperation, it is intriguing that the homo-oligomeric Hip molecule combines structural elements found in Hsc70 and other Hsc70/Hsp90-associated co-chaperones as well (7). For example, near the amino terminus Hip possesses multiple stretches of a degenerate 34-amino acid motif, so-called TPR repeats (27, 28). TPR repeats are also found in p60/Sti1 and the Hsp90-binding immunophilins CyP-40 and FKBP52 (25, 27, 29). Moreover, several repeats of the tetrapeptide GGMP, previously identified in cytosolic Hsp70 molecules as part of a regulatory motif (30, 31), are located close to the carboxyl terminus of Hip together with an additional p60/Sti1-related region (7). The evolutionary conservation suggests an importance of these domains for Hsc70/Hsp90 regulation and cooperation. A functional characterization of individual domains of the Hip protein was therefore initiated, resulting in the localization of the Hsc70-binding site of Hip and the identification of a domain required for homo-oligomerization. These findings are discussed with respect to the function of Hip in Hsc70/Hsp90-mediated protein biogenesis.

MATERIALS AND METHODS

Analysis of Hip Deletion Mutants Using the Yeast Two-hybrid System—Deletion mutants of Hip were constructed by subcloning polym-

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1 The abbreviations used are: Hip, heat shock protein; Hsc70, 70-kDa heat shock cognate protein; Hip, Hsc70-interacting protein; CyP, cyclophilin; FKBP, FK-506-binding protein; TPR, tetratricopeptide; MBP, maltose-binding protein; N-Hip, amino acids 1–256 Hip fragment; N’-Hip, amino acids 38–256 Hip fragment; MOPS, 4-morpholinepropane-sulfonic acid.
erge chain reaction-amplified fragments of the rat hip gene into pGAD424 (Clontech Laboratories, Inc.). Oligonucleotides used for amplification contained restriction sites suitable for in-frame subcloning of the hip fragments downstream of the GAL4 activation domain coding region. All polymerase chain reaction products were sequenced to confirm the wild-type character. The resultant constructs were introduced into yeast strain HF7c (Clontech Laboratories, Inc.), and expression of Gal4 activation-domain-Hip fusion proteins was investigated after growth of the corresponding transformants on synthetic dextrose minimal medium under selective conditions for 2 days (32). Following SDS-polyacrylamide gel electrophoresis of crude yeast extracts, fusion proteins were detected by immunoblotting using a monoclonal anti-Gal4 activation-domain antibody (Clontech Laboratories, Inc.) and the ECL system (Amersham Corp.). Solubility of fusion proteins was assessed by disruption of expressing cells in 20 mM HEPES-KOH, pH 7.4, 50 mM KCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.06% Triton X-100, followed by centrifugation at 5,000 × g for 10 min and a second centrifugation step of the resultant supernatant fraction at 100,000 × g for 30 min. HF7c cells expressing the chimeric Hip constructs were co-transformed with plasmid GAL4(bd)-Hsc70(1–383) encoding a fusion protein of the ATPase fragment of rat Hsc70 and the Gal4 DNA-binding domain (7). Interaction with the Hsc70 ATPase domain was analyzed as described previously (7).

Construction and Purification of MBP-Hip Fusion Proteins—To obtain the MBP proteins with the MBP, polymerase chain reaction-amplified fragments of the hip gene carrying a translational stop codon at their 3' ends were subcloned into vector pMAL-c2, which encodes a cytosolic form of MBP based on the bacterial maltE gene (New England Biolabs). In the corresponding fusion proteins the Hip portion is preceded by a vector-encoded 20-amino acid spacer sequence. pMAL-c2 fusion constructs were transformed into E. coli TG1, and expression of MBP-Hip fusion proteins was induced by addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. After induction for 1.5 h at 37°C, cells were collected and lysed in 20 mM HEPES-KOH, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride using a French pressure cell, followed by centrifugation at 35,000 × g for 30 min. The supernatant fraction was loaded onto an amylose column for affinity purification of the MBP-Hip fusion proteins according to the protocol of the manufacturer (New England Biolabs). As a nontagged control protein, purified MBP2α, which possesses the 20-amino acid spacer sequence (designated MBP throughout the text), was commercially obtained (New England Biolabs).

Biochemical Analysis of Hip-Hsc70 Interaction—The wild-type rat Hip protein, the N-Hip (amino acids 1–256) and the N'-Hip (amino acids 20–383) fragments, and the ATPase domain of bovine Hsc70 were expressed from corresponding pET plasmids in E. coli BL21(DE3) cells according to the protocol of the manufacturer (Novagen, Inc.). Hip, N-Hip, and N'-Hip were purified by ion-exchange and hydroxyapatite chromatography.4 Purification of the ATPase domain was performed as described (33), and the domain was immobilized on CNBr-activated Sepharose 4B at 10 mg of protein/ml of resin as recommended by the manufacturer (Pharmacia Biotech Inc.). Interaction of Hip, Hip fragments, and MBP-Hip fusion proteins with the ATPase domain was monitored by incubation of 150 µl of immobilized ATPase domain with 60 µg of purified protein in 500 µl of Buffer A (20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 2 mM MgCl2) containing 0.1 mM ADP. Incubation was performed at 4°C for 30 min prior to washing the resin with 15 volumes of Buffer A. Specifically retained protein was eluted by addition of 1 ml of Buffer A containing 1 mM ATP and incubation for 10 min at 30°C followed by addition of 1 ml of Buffer A containing 250 mM NaCl.

Miscellaneous—Recombinant DNA techniques were performed as described (32). Gel filtration was performed using a Superose 12 column (Pharmacia) equilibrated in 20 mM MOPS-KOH, pH 7.2, 50 mM KCl, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol. Protein concentrations were determined using the Bio-Rad Bradford assay reagent with bovine γ-globulin as the standard. Structure analysis was performed using the DNASTAR program (DNASTAR, Inc.).

RESULTS

By Employing the Yeast Two-hybrid System, the Hsc70-binding Site of Hip Was Mapped to a Domain Comprising Multiple TPR Repeats and Flanking Charged α-Helices—To identify structural elements required for binding of Hip to the ATPase domain of Hsc70, we took advantage of the yeast two-hybrid system (34). Different domains of the Hip protein were fused to the activation domain of the transcriptional activator Gal4, and the chimeric proteins were expressed in yeast strain HF7c in a soluble form (Fig. 1, A and B). Strain HF7c co-expressed the ATPase domain of rat Hsc70 (amino acids 1–383) fused to the Gal4 DNA-binding domain (7). Utilizing HIS3 as a Gal4-regulated reporter gene, interaction of the Hip deletion constructs with the ATPase domain was monitored following incubation of double transformants on histidine-free minimal medium. As previously reported, full-length Hip binds to the ATPase domain of Hsc70, resulting in a His+ phenotype in the two-hybrid assay (Fig. 1C) (7). Similarly, transformants carrying fusions of the Gal4 activation domain to amino acids 1–257 and 15–368 of Hip formed single-cell colonies on selective medium. In all cases, growth was dependent on the co-expression of the
ATPase domain construct (data not shown). Apparently, interaction with the ATPase domain is mediated by a region near the amino terminus of Hip (amino acids 15–257) and does not require the Hsc70- and Sti1-like domains located at the carboxyl terminus. Transformed cells carrying a construct lacking the three consecutively arranged TPR repeats (deletion of amino acids 130–215) did not display a His+ phenotype despite stable expression of the fusion protein (Fig. 1, AΔTPR). In addition, a positive growth phenotype was not observed when regions adjacent to the TPR repeats were deleted. Sequence analysis predicts an extended α-helical conformation for the regions flanking the TPR repeats, i.e. amino acids 45–112 and 215–256 (Fig. 2). Remarkably, the α-helical segment preceding the TPR repeats displays an accumulation of negatively charged amino acids (23 residues in the 67-amino acid stretch), resulting in a deduced isoelectric point of 3.8. In contrast, the amino acid 215–256 region contains predominantly positively charged amino acids (16 residues in the 41-amino acid stretch), with a deduced isoelectric point of 10.7. The domain of the Hip protein (amino acids 15–257) shown to mediate binding to Hsc70 thus contains as major structural elements three consecutively arranged TPR repeats preceded by an acidic α-helical segment and followed by a basic α-helix.

The Extreme Amino Terminus of Hip Is Required and Sufficient for Homo-oligomerization of Hip—Based on the results of the two-hybrid approach, mutant forms of the Hip protein were designed. Fragments of Hip comprising amino acids 1–256 (N-Hip) and 38–256 (N′-Hip) appeared of interest since they were affinity-purified on an amylose column. The fusion protein with a deduced molecular mass of 57 kDa eluted from a Superose 12 column in a defined peak at 10.0 ml, corresponding to a size of about 300 kDa (Fig. 4B). The amino terminus of Hip (amino acids 1–124) apparently induces and mediates homo-oligomerization. Furthermore, the data exclude an essential role of the TPR repeats for Hip-Hip subunit interaction since the Hip portion of the fusion protein does not constitute a complete TPR repeat.

Ironically, the amino terminus is also sufficient for homo-oligomerization, residues 1–124 of Hip were fused to the monomeric MBP (Fig. 4A). MBP-(1–124) was expressed in E. coli in a soluble form and was affinity-purified on an amylose column. The fusion protein with a deduced molecular mass of 57 kDa eluted from a Superose 12 column in a defined peak at 10.0 ml, corresponding to a size of about 300 kDa (Fig. 4B). The amino terminus of Hip (amino acids 1–124) apparently induces and mediates homo-oligomerization. Furthermore, the data exclude an essential role of the TPR repeats for Hip-Hip subunit interaction since the Hip portion of the fusion protein does not constitute a complete TPR repeat.

Hsc70 Interaction Is ATP- and Salt-dependent and Predominantly Mediated by the Positively Charged α-Helix Following the TPR Repeats—The purification of Hip fragments allowed us to biochemically verify interaction with the ATPase domain of Hsc70 observed in the two-hybrid assay. This appeared essential since Hip is part of a larger chaperone complex in the eukaryotic cytosol comprising several additional proteins apart from Hsc70 (7, 16, 17). Conceivably, interaction in the two-hybrid system could be mediated by additional components rather than through direct recognition of the ATPase domain by Hip. In addition, MBP fusion proteins possessing different portions of the amino acid 38–256 region of Hip were expressed and purified to further assess the role of the TPR repeats and
the flanking charged α-helices for binding of Hip to the Hsc70 ATPase domain (Fig. 4A).

Purified ATPase domain of bovine Hsc70 was immobilized on activated Sepharose and incubated with purified Hip, Hip fragments, and MBP-Hip fusion proteins (Fig. 5). Native Hip and the N-Hip fragment (amino acids 1–256) were quantitatively retained on the Hsc70 ATPase domain column in the presence of MgATP but did not nonspecifically bind to a Sepharose column lacking the domain. Consistent with previous observations demonstrating a reduced affinity of Hip for Hsc70 in the presence of MgATP (7), both proteins were partially eluted from the affinity resin by addition of MgATP (Fig. 5). Protein that remained bound to the resin under this condition was completely released by subsequent addition of 250 mM NaCl, revealing the importance of ionic interactions for binding of Hip to the ATPase domain. Salt-induced dissociation of Hip-containing chaperone complexes in reticulocyte lysate has in fact been reported (7, 16). The characteristics observed for binding of purified authentic Hip and the N-Hip fragment to the immobilized ATPase domain of Hsc70 thus correlate with the characteristics observed for stabilization and dissociation, respectively, of Hip-containing complexes in cytosolic extracts. Hence, interaction with the ATPase domain of Hsc70 mediated by the amino acid 1–256 portion of the Hip protein is apparently the main determinant for recruitment of Hip to chaperone complexes in the eukaryotic cytosol.

When the N’-Hip fragment (amino acids 38–256) was incubated with the ATPase domain, the protein was still significantly retained on the affinity resin compared with the control reaction using ovalbumin (Fig. 5). However, part of the protein was detected in the flow-through and subsequent wash fractions. The appearance of the fragment in these fractions may be due to the monomeric character of N’-Hip (Fig. 3B). Addition of MgATP reduced the affinity of N’-Hip for the ATPase domain, suggesting that the observed retention of N’-Hip reflects a specific interaction. Furthermore, the 38–256 portion of Hip mediated significant retention of a corresponding MBP fusion protein (MBP-(38–256), Fig. 5). The region of Hip comprising the TPR repeats and the flanking charged α-helices appears to be sufficient to mediate binding to the Hsc70 ATPase domain. Affinity chromatography thus confirms the localization of the Hsc70-binding site based on the two-hybrid approach.

The role of distinct structural elements within the 38–256 region of Hip was assessed utilizing additional MBP-Hip fusion proteins. The fusion proteins were expressed in E. coli in a soluble form (Fig. 4A) and could be affinity-purified on an amylose column, indicating their correct folding. A fusion construct comprising almost exclusively the negatively charged stretch preceding the TPR repeats did not interact with the immobilized ATPase domain (MBP-(38–124), Fig. 5). Moreover, only a minor amount of MBP-(38–218), carrying the negatively charged stretch and the three consecutively arranged TPR repeats, was retained on the affinity matrix in an ATP-dependent manner. In contrast, the positively charged α-helical stretch that follows the TPR repeats in the authentic Hip protein displayed a significant affinity for the immobilized
ATPase domain when fused to MBP (MBP-(216–256), Fig. 5). The binding behavior of MBP-(216–256) was comparable to that of the construct comprising the complete Hsc70-binding site (MBP-(38–256)) and was ATP- and salt-dependent. The data suggest that the ATPase domain is predominantly contacted through the positively charged α-helix following the TPR repeats.

**DISCUSSION**

Utilizing the yeast two-hybrid system and an *in vitro* affinity assay, we were able to identify the Hsc70-binding site of the eukaryotic co-chaperone Hip. The binding site is located close to the amino terminus of Hip and apparently comprises three consecutively arranged TPR repeats flanked by an acidic α-helical segment and a basic α-helix (Fig. 6). An involvement of TPR repeats in protein-protein interactions has previously been demonstrated (27, 28). In the case of Hip, however, the TPR repeats are not sufficient for binding to the Hsc70 ATPase domain (Figs. 1 and 5). In fact, contact of Hip with Hsc70 appears mediated primarily by the α-helix that follows the TPR repeats and displays an accumulation of positively charged residues (amino acids 216–256) (Figs. 2 and 5). The basic α-helix may form ionic interactions with the ATPase domain, explaining the observed salt sensitivity of Hip-Hsc70 complex formation (7, 16). A requirement of the TPR repeats of Hip for interaction with Hsc70 is revealed by the analysis of the ΔTPR construct in the two-hybrid approach. Although this construct is stably expressed and possesses the sequence elements required for homo-oligomerization (amino acids 1–124) and for contacting Hsc70 (amino acids 216–256), interaction with the ATPase domain was not observed (Fig. 1). Therefore, we propose that the TPR repeats are essential for the correct exposure of the basic α-helix within the Hip molecule. Similarly, a role for the structural integrity of the Hsc70-binding site of Hip has to be suggested for the acidic α-helical stretch preceding the TPR repeats since its deletion abolishes interaction in the two-hybrid assay (Fig. 1), but it does not display an affinity for the immobilized ATPase domain (Fig. 5).

Intriguingly, the architecture of the Hsc70-binding domain of Hip largely resembles that of the Hsp90-binding immunophilins FKB52 of rabbit and bovine CyP-40 as well as to TPR domains found in the yeast Sti1 protein.

![Functional Domains of the Hip protein and characterization of the TPR repeats of Hip.](image)
basic α-helix following the TPR repeats. It remains to be seen which structural element within the related region of CyP-40 specifically contacts Hsp90.

Hip and the immunophilins are components of the Hsc70-Hsp90 chaperone complex in the eukaryotic cytosol that mediates conformational changes, e.g. of steroid receptors and tyrosine kinases during signal transduction (7, 12–15). Yet, both proteins are associated with the complex at different stages of the Hsc70/Hsp90-mediated reaction. Whereas Hip, as a regulator of Hsc70, is involved in the early recognition and binding of substrates by the chaperone complex, the immunophilins interact with the chaperone-substrate complex at a later stage following the release of Hip (17). A chaperone-binding site of similar architecture may have evolved due to structural constraints imposed by Hsc70 and Hsp90, forming a specific contact with only one of the chaperone proteins. Such a recognition mechanism would enable the successive binding of different chaperone cofactors without necessarily inducing Hsc70-Hsp90 dissociation.

An important aspect of Hip function may relate to its homooligomeric structure. The amino terminus of the Hip protein (amino acids 1–124) is apparently required and sufficient to mediate the interaction of Hip subunits within the homo-oligomeric complex (Figs. 3B and 4B). Remarkably, monomeric Hip fragments retained the capacity to interact with the ATPase domain of Hsc70 (Fig. 5). Hence, homo-oligomerization does not appear to be essential for Hip/Hsp90 interaction, and each individual Hip subunit may provide an independent Hsc70-binding site. This is consistent with previous reports demonstrating the binding of at least two Hsc70 molecules per Hip homo-oligomer under in vitro conditions (7). In addition to its Hsc70 regulatory role, Hip may therefore fulfill a scaffolding function by holding multiple Hsc70 molecules in close proximity to an unfolded polypeptide substrate. Cooperative effects may nevertheless occur during the recognition of multiple Hsc70 molecules by the homo-oligomer and would explain the reduced binding affinity of the monomeric forms.

The identification of functional domains of the Hip protein will open new experimental approaches with which to analyze the role of Hip in the regulation of Hsc70 activity. This should contribute to our understanding of how molecular chaperones mediate conformational changes during protein folding and signal transduction in the eukaryotic cytosol.

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