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Mutational Analysis of the hsp70-Interacting Protein Hip

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The hsp70-interacting protein Hip participates in the assembly pathway for progesterone receptor complexes. During assembly, Hip appears at early assembly stages in a transient manner that parallels hsp70 interactions. In this study, a cDNA for human Hip was used to develop various mutant Hip forms in the initial mapping of functions to particular Hip structural elements. Hip regions targeted for deletion and/or truncation included the C-terminal region (which has some limited homology with *Saccharomyces cerevisiae* Sti1 and its vertebrate homolog p60), a glycine-glycine-methionine-proline (GGMP) tandem repeat, and a tetratricopeptide repeat (TPR). Binding of Hip to hsp70's ATPase domain was lost with deletions from the TPR and from an adjoining highly charged region; correspondingly, these Hip mutant forms were not recovered in receptor complexes. Truncation of Hip's Sti1-related C terminus resulted in Hip binding to hsp70 in a manner suggestive of a misfolded peptide substrate; this hsp70 binding was localized to the GGMP tandem repeat. Mutants lacking either the C terminus or the GGMP tandem repeat were still recovered in receptor complexes. Truncations from Hip's N terminus resulted in an apparent loss of Hip homo-oligomerization, but these mutants retained association with hsp70 and were recovered in receptor complexes. This mutational analysis indicates that Hip's TPR is required for binding of Hip with hsp70's ATPase domain. In addition, some data suggest that hsp70's peptide-binding domain may alternately or concomitantly bind to Hip's GGMP repeat in a manner regulated by Sti1-related sequences.

The assembly and maintenance of functionally mature progesterone receptor (PR) complexes require a complex, highly ordered pathway of protein interactions involving multiple components of the cytoplasmic molecular chaperone machinery (9, 12). An early participant in this pathway is a protein with a size estimated at 48 to 50 kDa from its mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9, 12). Antibodies against partially purified rabbit p48 were developed and used to clone a cDNA from a human HeLa expression library (7). In contrast to the size estimated from the mobility of p48 in SDS-PAGE, the human cDNA encodes a 41-kDa protein, but the protein product from this cDNA migrates identically to the PR-associated protein (7). Independently, a cDNA encoding a 41-kDa protein was isolated from a rat cDNA library by virtue of the protein's ability to interact with the ATPase domain of hsc70 in a *Saccharomyces cerevisiae* two-hybrid selection system (3). The protein was termed Hip to denote it as an hsp70-interacting protein. Since the rat and human cDNAs are over 90% identical, these are the presumptive species homologs for Hip, a term adopted here in place of p48.

It has been shown that rat and human Hip both interact with hsp70 in an ADP-sensitive manner (3, 7), and it has been proposed (3) that this interaction requires a DnaJ/hsp40 protein in a catalytic manner to promote hsp70-Hip binding. The possible involvement of DnaJ in hsp70-Hip complex formation may be related to earlier observations (1, 5) that the yeast DnaJ homolog YdjI is required for steroid receptor function in yeast cells, even though DnaJ homologs have not been identified as a major component of steroid receptor complexes.

In an effort to better understand Hip's interactions with hsp70, mutational analysis of a cDNA encoding human Hip was used to create a variety of truncated and internally deleted protein products. The activities of the mutant proteins were compared with that of wild-type (WT) Hip to map functionally distinctive Hip regions. The results from these studies are presented here.

MATERIALS AND METHODS

Preparation of Hip mutants. In vitro expression of the WT Hip protein was encoded by a 1.4-kb human Hip cDNA inserted into the pSPUTK (Stratagene) in vitro expression plasmid (7). All Hip mutant constructs were derived from the WT Hip-pSPUTK parental plasmid. In the construction of several mutant plasmids, site-directed mutagenesis was used to introduce convenient restriction sites. Except as noted, the double-stranded, site-directed mutagenesis Chameleon kit (Stratagene) was used to introduce the restriction sites.

A plasmid encoding the amino-terminal truncation mutant C-99 was produced by introducing an *Nco*I restriction site at the codon for Met-99 in Hip-pSPUTK's open reading frame (ORF). The mutated plasmid was digested with *Nco*I to remove sequences between the original *Nco*I 5′ insertion site (corresponding to the WT initiation codon) and the introduced *Nco*I site. The digest products were separated by gel electrophoresis, and the plasmid-containing fragment was religated. A plasmid encoding the C-15 mutant Hip product was similarly prepared, except an *Nco*I site was introduced at the codon for Met-15 in Hip-pSPUTK by using the Quikchange site-directed mutagenesis kit (Stratagene).

In preparing plasmids encoding C-terminal truncation products (N-303, N-226, and N-148), a unique *Pvu*II site in the 3′ untranslated region of Hip-pSPUTK was used. For the plasmid encoding N-303, Hip-pSPUTK was double digested with *Sph*I, which cuts at a unique site overlapping codon 304, and *Pvu*II, a blunt-cutting restriction enzyme. The 3′ overhang from *Sph*I digestion was trimmed by T4 polymerase treatment to create a blunt end, and the plasmid-containing product was religated by blunt-end ligation. The resulting construct contained a termination codon at position 304.

For the plasmid encoding N-226, Hip-pSPUTK was double digested with *Avr*II, which recognizes a unique site at codon 226, and *Pvu*II. To create a blunt end, the 5′ overhang from the *Avr*II digest was filled in by treatment with Klenow DNA polymerase I. The plasmid-containing product was religated by blunt-end ligation. The resulting protein product (N-226) contains a terminal Ser at position 227.

For the plasmid encoding N-148, Hip-pSPUTK was double digested with *Msc*I, a blunt-cutting enzyme recognizing a unique restriction site overlapping codon 148, and *Pvu*II. Blunt-end ligation of the plasmid-containing fragment created an ORF encoding Hip amino acids 1 to 148 fused with 14 amino acids (QAFYSG LFLGYSFR) encoded by sequences in the 3′ untranslated region of Hip cDNA.

For the plasmid encoding N-276, Hip-pSPUTK was digested with *Sac*I, which cuts both at a unique site overlapping codon 276 of Hip's ORF and in the 3′ polylinker of pSPUTK. Religation of the digested plasmid resulted in an ORF

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encoding amino acids 1 to 276 of Hip fused with four amino acids (RIHR) encoded by pSPUTK sequences.

Internal deletion mutants were created as follows. A plasmid encoding ΔTPR was constructed by introducing an *Avr*II restriction site at codon 117 of Hip-pSPUTK. Taking advantage of the preexisting *Avr*II site at codon 226, the mutated Hip-pSPUTK was digested with *Avr*II, and the digestion products were separated by gel electrophoresis. The large plasmid-containing product was religated to create the deletion mutant.

Similarly, to create a plasmid encoding ΔGGMP, an *Sph*I site was introduced at codon 281 of Hip-pSPUTK, which already contained a unique *Sph*I site overlapping codon 304. The mutated plasmid was digested with *Sph*I, and the plasmid-containing fragment was religated. To create the plasmid encoding Δcharge, an *Spe*I site was introduced at codon 282 of Hip-pSPUTK. The mutated plasmid was digested with *Avr*II and *Spe*I to remove DNA for the intervening codons 227 to 281. The resulting compatible sticky ends in the plasmid-containing fragment were religated.

In vitro expression and coprecipitation of Hip forms. Radiolabeled Hip protein forms were prepared by expressing WT and mutant plasmid constructs in an in vitro transcription-translation system (TnT kit; Promega) in the presence of [^{35}S]methionine. To measure protein-protein interactions with Hip forms, aliquots of separate synthesis mixtures containing equivalent amounts of incorporated radioactivity were added to normal rabbit reticulocyte lysate (RL). hsp70 association was measured by coprecipitation of labeled Hip forms with an hsp70 immunoaffinity resin. Monoclonal antibody BB70 (11) was preadsorbed to protein G-Sepharose (Pharmacia), and the resin was added to RL mixtures. The proportions of ingredients in each mixture were 15 μg of BB70 on a 10-μl resin pellet in a total RL volume of 100 μl. Samples were incubated at 30°C for 30 min and quenched with 1 ml of cold WB (50 mM Tris [pH 7.4], 10 mM monothioglycerol, 1% Tween 20). Each sample was divided and washed with either WB alone or WB plus 0.4 M NaCl to promote dissociation of normal Hip-hsp70 interactions (7). Resin-bound proteins were separated by SDS-PAGE and visualized by Coomassie blue staining and autoradiography.

To measure the abilities of Hip forms to participate in the PR assembly pathway, in vitro assembly of PR complexes was carried out as previously described (9, 12). Briefly, PR from chick oviduct cytosol was bound to an anti-PR immunoaffinity resin. The PR-resin complexes were added to RL and incubated at 30°C for 30 min to establish steady-state assembly conditions (9). For the present experiments, assembly mixtures were supplemented with the hsp90-binding drug geldanamycin (20 μg/ml) to enhance recovery of early PR complexes containing Hip (12). Aliquots of radioactively labeled Hip synthesis mixtures were added separately to PR assembly reaction mixtures prior to initiation of assembly. The PR-resin complexes were washed and separated by SDS-PAGE. Proteins were visualized by Coomassie blue staining and by autoradiography.

Expression of hsp70 ATPase domain and interactions with Hip forms. To prepare DNA encoding just the ATPase domain of hsp70, the desired product was synthesized by PCR amplification. The template was a rat hsc70 cDNA (8) subcloned into the pSPUTK cell-free expression plasmid (Stratagene). The forward primer (5′-ATCTACCATGGCTAAGGG) overlapped an *Nco*I restriction site that had been previously engineered at the initiation codon for hsc70. The reverse primer (5′-GGTCTGCAGCTAGTAGTCTCCAGATAGGGC) was complementary to codons 379 to 383 of hsc70 but introduced a termination codon at position 384 and incorporated a *Pst*I restriction site immediately downstream from the termination codon. The PCR used 50 ng of hsc70/pSPUTK for the template and 50 pmol of each oligonucleotide primer in a 50-μl reaction volume. Amplification with Deep Vent Exo$^-$ polymerase (New England Biolabs) was performed with 25 cycles of denaturation at 95°C (1 min), annealing at 42 to 56°C (1 min), and extension at 75°C (66 s) with a Robocycler Gradient Cycler (Stratagene). The 1.2-kb product was purified on a Qiagen Quick-Spin column, digested with *Nco*I-*Pst*I, and subcloned into the pSPUTK expression plasmid. The correct DNA sequence for the PCR product was verified by automated sequencing. Following expression of the DNA in a cell-free transcription-translation system (TnT lysate; Promega) in the presence of [^{35}S]methionine, an appropriate 40-kDa product was detected by SDS-PAGE and autoradiography.

Interaction between the hsc70-ATPase domain and Hip forms was determined as follows. With the exception of N-276, wild-type and mutant Hip cDNAs were subcloned from the pSPUTK expression plasmid into the pET-28a bacterial expression plasmid (Novagen). Hip cDNA was digested with *Nco*I, which cuts at the beginning of Hip's ORF, and filled in by Klenow treatment to create a blunt end. The Hip cDNA was isolated from pSPUTK by *Eco*RI digestion and gel electrophoresis. To prepare pET-28a for Hip insertion, the plasmid was digested with *Nhe*I, filled in by Klenow treatment to create a blunt end, and then digested with *Eco*RI. The Hip cDNA was inserted into the plasmid, creating an ORF that incorporates a 21-amino-acid fusion at Hip's N terminus, including a poly-His tag to facilitate purification of Hip products. Hip-pET28 constructs were used to transform BL21 bacterial cells, and expression of Hip forms was induced with isopropyl-β-D-galactopyranoside. His-tagged Hip forms were purified from bacterial cell extracts by binding to Ni^{2+} affinity resin (Novagen). His-resin complexes (approximately 10 μg of protein on a 10-μl resin pellet per sample) were then incubated at 30°C for 30 min in RL (100-μl total volume per sample) supplemented with radioactively labeled hsc70-ATPase domain. Each sample was then divided and washed with either WB alone or WB plus 0.4 M NaCl. Complexes were separated by SDS-PAGE and visualized by Coomassie blue staining and autoradiography.

RESULTS

Development and expression of Hip mutants. Site-directed mutagenesis was used to prepare mutant human Hip cDNAs for production of truncated or internally deleted Hip proteins. Figure 1A presents diagrams of the WT and mutant Hip forms prepared. Four structurally distinctive regions that were targeted for mutagenesis are highlighted. These regions (and the corresponding mutants) are (i) the tetratricopeptide repeat (TPR) region at approximately amino acids 100 to 200 (ΔTPR), (ii) a 50-amino-acid region enriched in charged residues (Δcharge), (iii) a 33-amino-acid stretch containing degenerate tandem repeats of the sequence glycine-glycine-methionine-proline (ΔGGMP), and (iv) the C terminus (N-303), which has some homology (3) with the C termini of yeast Sti1 (6) and its human homolog p60 (4) (an alternate name for p60—hsp90/hsp70-organizing protein [Hop]—has been proposed [2] and will be used by us in future publications). Since p60 is also an hsp70-interacting protein (2, 11), Hip's C-terminal region was of interest, even though deletion of this region from p60 did not alter its ability to bind hsp70 (2). In addition to these four mutants, several additional carboxy- or amino-terminal truncation mutants were generated (Fig. 1A).

Mutant cDNAs were expressed in a cell-free transcription-translation system in the presence of [^{35}S]methionine, and the labeled protein products were examined by SDS-PAGE and autoradiography (Fig. 1B). It is apparent from the SDS-PAGE profiles that the WT and all mutant proteins except the N-terminal truncation mutant C-99 migrate with apparent molecular masses about 10,000 Da greater than that predicted from amino acid sequences alone (Table 1). Since C-99 lacked the large differential between the predicted size and the electrophoretically estimated size, this property appears to reside in the N-terminal 100 amino acids of Hip. However, as discussed below, a mutant lacking just the amino-terminal 14 amino acids (C-15) migrates almost identically to WT Hip.

Hip sequences required for homo-oligomerization. Höhfeld et al. (3) reported that rat Hip appears to exist as a tetramer as judged by gel filtration patterns. Human Hip mutants were examined for their ability to form homo-oligomeric complexes, as shown in Fig. 2. Radioactively labeled Hip forms were prepared by in vitro expression. Each synthesis mixture was adjusted to 0.4 M NaCl to dissociate heteromeric complexes with hsp70, and equal aliquots of each were supplemented with either the homobifunctional cross-linker bis(sulfosuccinimidyl) suberate (2.5 mM final concentration) or an equal concentration of the related monofunctional compound sulfosuccinimidyl acetate. According to the manufacturer (Pierce), these compounds react preferentially with the ε-amine of lysine side chains. After 30 min at room temperature, reactions were quenched with excess Tris, samples were separated by SDS-PAGE, and autoradiographs were prepared. All of the Hip forms examined for Fig. 2A were found to form larger complexes in the presence of the cross-linker. The complexes appearing in the cross-linked WT sample (Fig. 2A, lane 1) have estimated sizes of approximately 100 kDa and, for the more diffuse, more slowly migrating material, 120 to 180 kDa. Thus, it appears that Hip forms dimers and perhaps trimers or tetramers, with the caveat that cross-linkage may alter the gel mobility of Hip complexes (see Fig. 2B). On the basis of densitometry of autoradiographs, cross-linked and control lanes contain approximately equivalent levels of radioactively labeled Hip, indicating the absence of large cross-linked aggre-

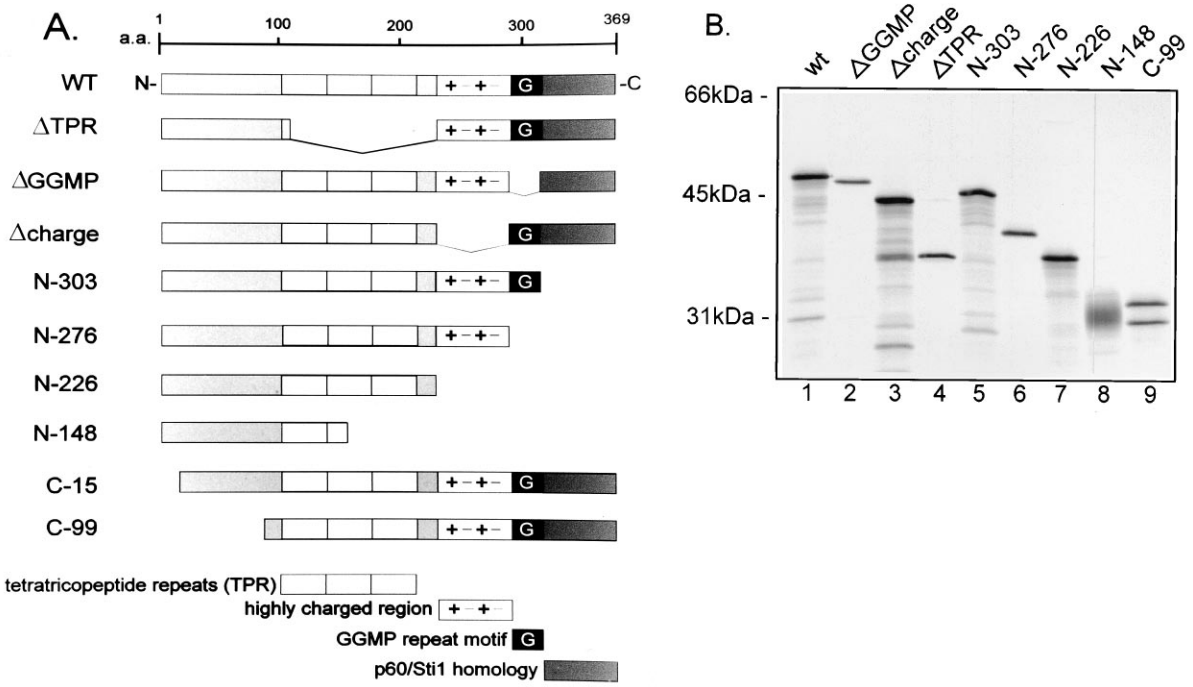

FIG. 1. Hip mutant forms. Several plasmids encoding truncation and deletion mutants of human Hip were generated. (A) Diagrams of Hip forms, highlighting structurally distinct regions. a.a., amino acid. (B) Expression of Hip forms. WT and mutated Hip cDNAs were expressed in vitro in the presence of [^{35}S]methionine. Aliquots of the synthesis mixtures were separated by SDS-PAGE and autoradiographed. The lower band appearing at an approximately equal amount with mutant C-99 (lane 9) is probably a product whose synthesis initiates from a double Met codon at positions 110 and 111 of WT Hip.

gates that are incapable of entering the gel matrix, but higher-order homo-oligomeric Hip complexes that are incompletely cross-linked may normally exist.

Since endogenous rabbit Hip in the RL synthesis mixtures is more abundant than the small amount of radioactively labeled human Hip forms produced, it is assumed that the cross-linked complexes observed by autoradiography reflect a single subunit of the labeled Hip associated with one or more subunits of endogenous, WT Hip. With this in mind, the C-terminally truncated and internally deleted Hip forms examined for Fig. 2A display a similar pattern of cross-linking, with reductions in the sizes of cross-linked complexes corresponding to the size of the labeled component.

In contrast to the case for the mutants examined for Fig. 2A, N-terminal truncation apparently prevents Hip homo-oligomerization (Fig. 2B). A truncation of 14 amino acids from Hip's N terminus (C-15) resulted in minimal formation of higher-order cross-linked complexes. In fact, the cross-linker causes a paradoxical increase in the mobility of C-15 in SDS-PAGE (compare lanes 3 and 4 of Fig. 2B). The explanation for this observation may lie in nearby downstream sequences responsible for retarded gel mobility. Note that the migration of un-cross-linked C-15 (Fig. 2B, lane 4) is almost identical to that of WT Hip (lane 2), suggesting that C-15, like most other Hip forms, is about 10,000 Da greater in molecular mass than predicted from its amino acid sequence (Table 1). It was shown above, however, that C-99 more closely follows its expected mobility (Fig. 1B, lane 9). Within the additional sequences truncated from C-99 compared with C-15, amino acids 50 to 100 contain 26 charged residues, 6 of which are lysines. Therefore, C-15's reduced mobility in the presence of the cross-linker (Fig. 2B, lane 3) may result from modifications in this charge-rich region that disrupt a structural feature causing retarded gel mobility. In Hip forms that retain homo-oligomerization, an increased mobility related to cross-linking would not be as apparent (Fig. 2A).

TABLE 1. Size comparison of Hip forms predicted from amino acid sequences or estimated from SDS-PAGE mobilities

Hip form	Size (kDa)		Difference (kDa)
	Predicted	SDS-PAGE	
WT	41.3	49.3	8.0
ΔTPR	29.1	37.4	8.3
ΔGGMP	38.6	48.9	10.3
ΔCharge	35.0	45.7	10.7
N-303	34.6	44.4	9.8
N-276	31.8	40.3	8.5
N-226	25.5	36.9	11.4
N-148	16.7	29.5	12.8
C-15	39.6	49.2	9.6
C-99	30.1	31.2	1.1

Further suggesting the importance of Hip's N terminus in self-associations, an observation not reliant on cross-linking was made (Fig. 2C). Bacterial expression plasmids were constructed by using the pET-30a vector to place a polyhistidine fusion tag at the N termini of Hip forms. In creating these constructs, Hip-encoding cDNA was inserted in frame at an *Nco*I site immediately downstream from vector sequences encoding the polyhistidine fusion (a total of 44 amino acids fused to the N termini of Hip sequences). Judging from the products produced in vitro with a combined transcription-translation system, the Met codon at position 45 appears to function efficiently for initiating protein synthesis. With gel separations of total synthesis mixtures (Fig. 2C), there are two major radiolabeled products for WT Hip (lane 1), mutant C-15 (lane 3), and C-28, an additional N-terminal truncation mutant lacking

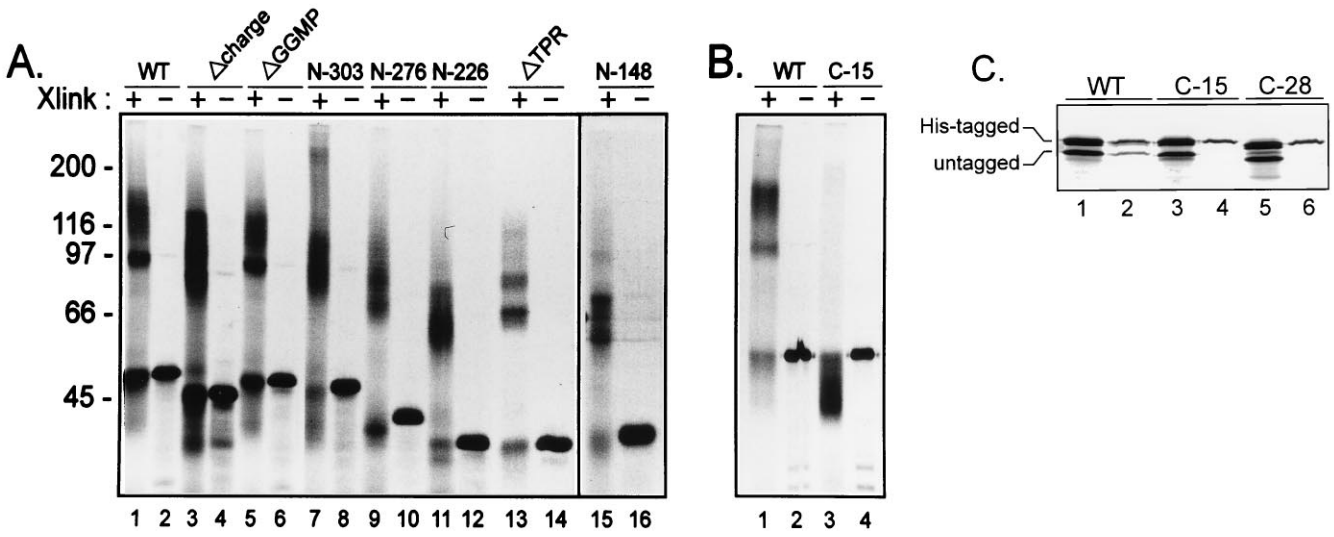

FIG. 2. Analysis of Hip homo-oligomerization. Hip forms were synthesized in vitro in the presence of [^{35}S]methionine. Aliquots of each synthesis mixture were adjusted to 0.4M NaCl to dissociate Hip-hsp70 complexes, treated with the cross-linker bis(sulfosuccinimidyl)suberate or non-cross-linking sulfosuccinimidyl acetate as indicated above the lanes (Xlink), and analyzed by SDS-PAGE and autoradiography. (A) Hip forms displaying high levels of cross-linking. Mutant N-148 (lanes 15 and 16) has a faster mobility than other Hip forms, but the image for N-148 samples was juxtaposed with larger Hip forms. (B) In a separate experiment, WT and C-15 forms of Hip were metabolically labeled, treated with bis(sulfosuccinimidyl)suberate or sulfosuccinimidyl acetate, separated by SDS-PAGE, and visualized by autoradiography. (C) Coelution patterns for His-tagged and nontagged Hip forms on Ni^{2+}-agarose. His-tagged WT Hip and N-terminal truncation mutants C-15 and C-28 were radiolabeled by expression in TnT lysate and examined for their abilities to bind an Ni^{2+} affinity resin. For each assay, 5 μl of the radiolabeled synthesis mixture was added to a 5-μl pellet of Ni^{2+}-agarose in 100 μl of binding buffer. After incubation on ice for 30 min, the resin pellets were washed and extracted into SDS sample buffer. Total synthesis mixtures (lanes 1, 3, and 5) or resin-adsorbed proteins (lanes 2, 4, and 6) were separated by SDS-PAGE and autoradiographed. The upper band in the total synthesis mixtures represents the His-tagged product, while lower bands appear to be products lacking the N-terminal His tag because of translation initiation from a downstream Met codon. (See Results for a further explanation.)

amino acids 1 to 27 of Hip (lane 5). The probable explanation for the smaller product in each sample is a relative lack of discrimination between nearby Met codons used for translation initiation in the cell-free system. The first Met immediately precedes the polyhistidine fusion, while the second, Met-44, is encoded by DNA sequences containing an *Nco*I site and thus encoding a good Kozak consensus sequence for efficient translation initiation. Assuming that this is correct, then each synthesis mixture contains a combination of radiolabeled Hip forms containing or lacking an N-terminal polyhistidine tag.

When the synthesis mixtures were subjected to affinity binding with Ni^{2+}-agarose, different recovery patterns were observed for mutant Hip forms compared with WT Hip. Both the larger His-tagged and smaller nontagged WT products were recovered on affinity resin (Fig. 2C, lane 2). On the other hand, nontagged mutant forms were not recovered along with the His-tagged mutant forms (Fig. 2C, lanes 4 and 6). In other words, the WT nontagged product appears to oligomerize with the corresponding His-tagged product and thus copurifies on Ni^{2+}-agarose; the failure of nontagged mutant forms to copurify supports the suggestion from cross-linking data (Fig. 2A and B) that N-terminal sequences required for oligomerization are lacking in these mutants.

Hip sequences required for hsp70 interactions. Since Hip is an hsp70-binding protein, Hip mutants were examined for their ability to interact with hsp70 (Fig. 3). WT and mutant proteins were metabolically labeled by in vitro expression of cDNAs in the presence of [^{35}S]methionine. Aliquots of each synthesis mixture were added to normal RL and incubated at 30°C to promote the formation of hsp70-Hip complexes. The RL mixtures were immunoprecipitated with the anti-hsp70 monoclonal antibody BB70, and precipitated complexes were examined by SDS-PAGE and autoradiography. Elevated ionic strength does not typically dissociate hsp70 from misfolded peptide substrates, in contrast, Hip binding to hsp70 is normally salt sensitive (7), presumably reflecting the distinct interaction of Hip with hsp70's ATPase domain rather than with hsp70's peptide-binding domain (3). Therefore, to distinguish normal Hip-hsp70 interactions from hsp70 binding to potentially misfolded Hip forms, aliquots of each immunoprecipitate were washed at elevated ionic strength.

At lower ionic strength, several proteins coprecipitate with hsp70 (Fig. 3A, upper panel). The major hsp70-associated proteins are hsp90 and p60, and their mutual interactions have been characterized in earlier reports (2, 11). Endogenous, unlabeled rabbit Hip also coprecipitates with hsp70-BB70 complexes, but it is not apparent in the Coomassie blue-stained gel because of its comigration with the BB70 heavy chain. As the autoradiographic results illustrate (Fig. 3A, lower panel), radiolabeled WT and some mutant Hip forms coprecipitated with hsp70 in a salt-sensitive manner. As judged by densitometry of bands on the autoradiographs, mutant forms ΔGGMP, N-276, and C-99 were recovered with hsp70 in a pattern similar to that for WT Hip, although at only 50 to 70% of the WT level. Hip mutants lacking the TPR region (ΔTPR [Fig. 3A, lane 7] and N-148 [Fig. 3A, lane 15]) were recovered in hsp70 complexes at less than 3% of the WT level. In two other mutants retaining the TPR region but lacking the adjacent highly charged region (Δcharge [Fig. 3A, lane 5] and N-226 [Fig. 3A, lane 13]), recoveries of the mutants were reduced by approximately 90% relative to that of WT Hip.

Unexpectedly, N-303, which lacks the C-terminal p60-homologous sequence but contains the GGMP region, displayed enhanced hsp70 binding (Fig. 3A, lane 9) that was largely insensitive to elevated ionic strength (lane 10). Following low- or high-salt washes, recoveries of N-303 were 150 and 125%, respectively, of the recovery of WT Hip with low-salt washes. The inability of salt to dissociate the interaction between N-303

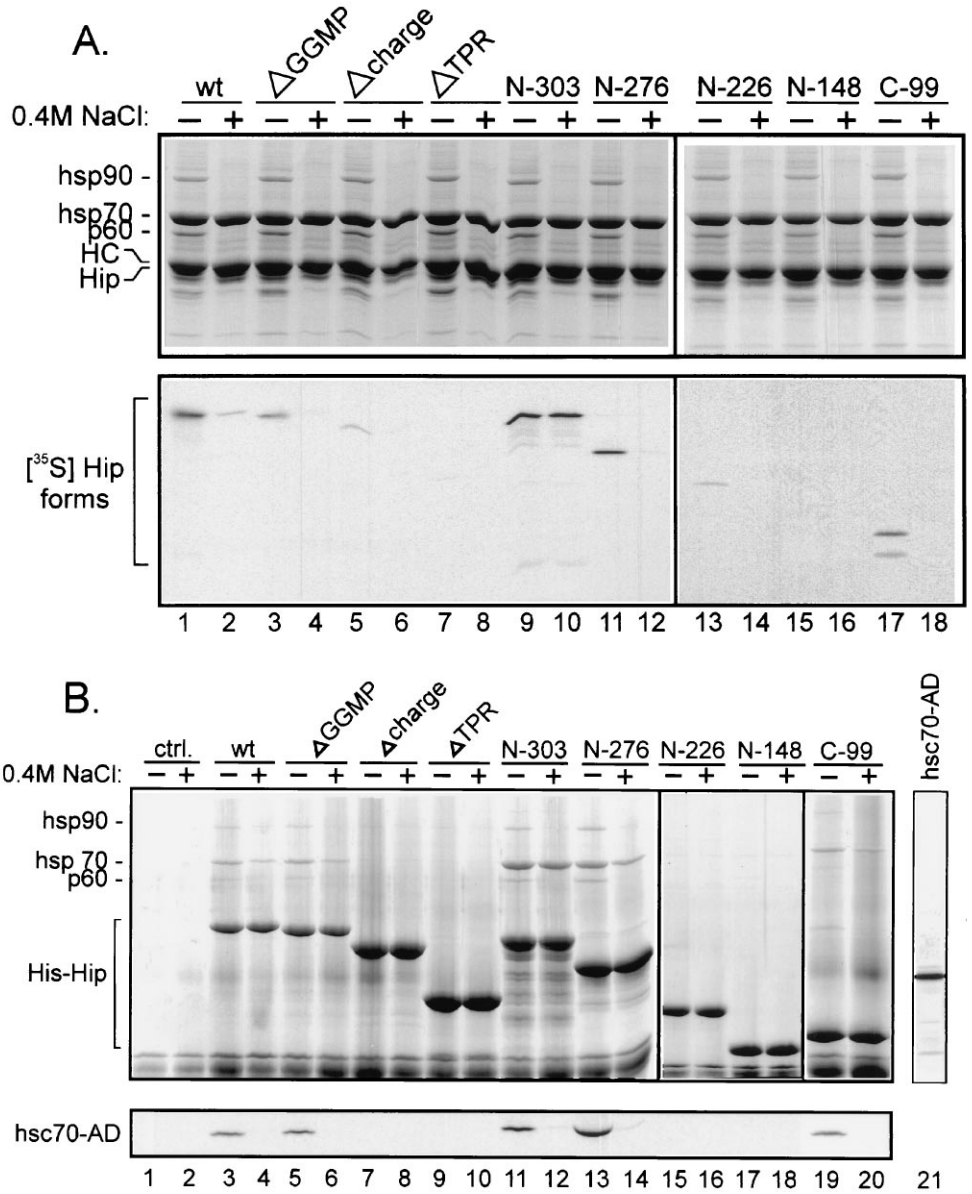

FIG. 3. (A) Interaction of Hip forms with full-length hsp70. Hip forms were metabolically labeled in vitro, added to normal RL in the presence or absence of 0.4 M NaCl, and immunoprecipitated with an hsp70 immunoaffinity resin, BB70-protein G-Sepharose. Components coprecipitating with hsp70 were separated by SDS-PAGE and visualized by Coomassie blue staining (upper panel) and autoradiography (lower panel). HC, heavy-chain band of monoclonal antibody BB70. (B) Interaction of Hip forms with the ATPase domain of hsc70 (hsc70-AD). His-tagged Hip forms were synthesized in bacteria and bound to Ni^{2+}-agarose affinity resin. To promote assembly of Hip-hsp70 complexes, aliquots of each resin, or a control resin lacking bound Hip (ctrl.) (lanes 1 and 2), were incubated with RL containing radiolabeled hsc70-AD. After being washed in either the absence or presence of 0.4 M NaCl, samples were separated by SDS-PAGE and visualized by Coomassie blue staining (upper panel, lanes 1 to 20) and by autoradiography (lower panel). N-227 and N-148 (lanes 15 to 18) and C-99 (lanes 19 and 20) samples were resolved on separate gels. For illustrative purposes, an aliquot of the labeled hsc70-AD synthesis mixture was separated by SDS-PAGE and autoradiographed (lane 21).

and hsp70 suggests that hsp70 may be binding N-303 in a manner distinct from that of WT Hip.

Since it was shown (3) that Hip normally binds the ATPase domain of hsc70 and not its C-terminal peptide-binding domain (14), we further examined N-303 and other Hip forms for their ability to interact with a rat hsc70 C-terminal truncation mutant (hsc70-AD) containing only the ATPase domain (Fig. 3B). In this assay, His-tagged recombinant Hip forms were isolated from bacterial extracts on Ni^{2+}-agarose. The Hip-resin complexes were added to RL containing radioactively labeled hsc70-AD and incubated at 30°C to promote Hip-hsp70 interactions. Resin complexes were washed in either low- or high-ionic-strength buffer, separated by SDS-PAGE, Coomassie blue stained (Fig. 3B, upper panel) to show the total proteins recovered with His-tagged Hip forms, and autoradiographed (Fig. 3B, lower panel) to reveal the presence of coprecipitating radiolabeled hsc70-AD.

The results in Fig. 3B are consistent with and extend those in Fig. 3A. The full-length hsp70, hsp90, and p60 bands in Coomassie blue-stained samples (Fig. 3B, upper panel) are endogenous RL proteins adsorbed from the hsc70-AD synthesis mixture. Densitometric measurements of full-length hsp70 recovered with various Hip forms show a pattern of binding similar to that observed in Fig. 3A. (For quantitative analysis of

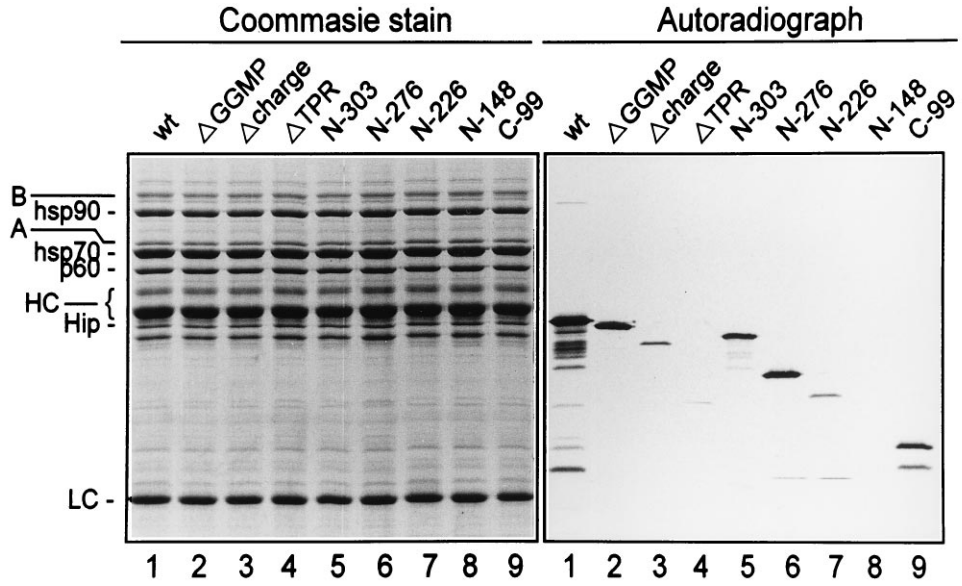

FIG. 4. Ability of Hip forms to enter assembly complexes with PR. Aliquots of metabolically labeled Hip forms containing approximately equivalent amounts of incorporated radioactivity were added separately to in vitro PR assembly mixtures. The RL medium for PR assembly was supplemented with 20 μg of geldanamycin per ml of RL to enhance recovery of early PR complexes containing Hip (12). Washed PR-resin complexes were separated by SDS-PAGE and visualized by Coomassie blue staining and by autoradiography. A and B, A and B forms of PR; HC and LC, heavy- and light-chain bands, respectively, of anti-PR monoclonal antibody PR22; Hip, endogenous rabbit Hip.

these samples, densitometric measurements were normalized to account for different molar quantities of His-tagged Hip forms in each sample.) Mutants other than N-303 containing both the TPR and highly charged regions (ΔGGMP, N-276, and C-99) bind full-length hsp70 at 100 to 200% of the WT level, and this binding is largely dissociable (50 to 80%) with high-salt washes. Mutants lacking either or both of the TPR and charged regions (Δcharge, ΔTPR, N-227, and N-148) display negligible binding to full-length hsp70.

The recovery of radiolabeled hsc70-AD with Hip forms (Fig. 3B, lower panel) has a clear, salt-sensitive pattern. hsc70-AD association with Hip forms requires both the TPR and highly charged regions, since no binding of hsc70-AD is observed with Δcharge, ΔTPR, N-227, or N-148. In Hip forms for which hsc70-AD binding is observed (WT, ΔGGMP, N-303, N-276, and C-99), high-salt washes efficiently eliminate binding.

With mutant N-303, two patterns of hsp70 interaction are apparent. Binding of full-length hsp70 to N-303 is unaffected by salt (Fig. 3B, upper panel; compare lanes 11 and 12), although the hsp90 and p60 bands in the complex are dissociated by a high salt concentration. However, in contrast to full-length hsp70, binding of hsc70-AD is completely eliminated by the high salt concentration (Fig. 3B, lower panel; compare lanes 11 and 12). This dual behavior of N-303 toward hsp70 could be explained if deletion of Hip's C-terminal tail makes the GGMP repeat or a nearby upstream sequence a target for binding by hsp70's peptide-binding domain. Note that with N-276, in which both the C-terminal region and the GGMP repeat are absent, high-salt washes remove over 75% of full-length hsp70 associated with the complex (Fig. 3B, upper panel; compare lanes 13 and 14). In the alternate coprecipitation assay with immunoisolated hsp70 complexes (Fig. 3A, lanes 11 and 12), binding of N-276 to hsp70 is reduced by over 90% with high-salt washes.

The interactions illustrated in Fig. 3 all resulted from incubations in RL. Previous reports (3, 7) have indicated that Hip-hsp70 interactions are enhanced in the presence of ADP, although they may not be strictly dependent on ADP. For the studies shown, incubations were carried out at 30°C for 30 min in RL lacking an ATP-regenerating system. In a separate study using these conditions (10), endogenous ATP levels were reduced over 30 min from 2 to 3 mM to less than 0.5 mM, presumably with a corresponding increase in ADP levels as ATP was hydrolyzed. When the assays shown in Fig. 3 were repeated with an ATP-regenerating system included, the recovery of hsp70 with Hip forms was decreased but by less than 50% (results not shown). In one study (3), Hip-hsp70 interactions were established in a DnaJ-dependent manner by using purified proteins. However, we were previously unable to obtain heterocomplexes with purified Hip and hsp70 (7) and have also been unable to obtain binding with the addition of recombinant Hdj1 to the purified system (results not shown). It appears that factors or conditions unknown to us but present in RL are required for efficient binding of Hip to hsp70. Therefore, we have not been able to test interactions of Hip mutants with hsp70 in a purified system where nucleotides could be selectively included. Because of the apparent involvement of other factors in establishing Hip-hsp70 complexes, the requirement for Hip's TPR and adjacent highly charged region for efficient binding may be due to hsp70's ATPase domain interacting directly with this region of Hip or to a requirement for these regions in interacting with unidentified assembly factors.

Ability of Hip forms to enter PR complexes. As a further functional measure, Hip mutants were examined for their ability to participate in the PR assembly pathway (Fig. 4). RL was supplemented with a radioactively labeled synthesis mixture for each of the Hip forms prior to addition of immunoisolated PR resin complexes. Approximately equivalent amounts of incorporated radioactivity for each Hip form were added. To enhance recovery of early PR complexes containing Hip, RL was supplemented with the hsp90-binding drug geldanamycin, which blocks formation of mature PR complexes (12). After PR assembly reactions, resins were washed, and bound components were separated by SDS-PAGE. The Coomassie blue-

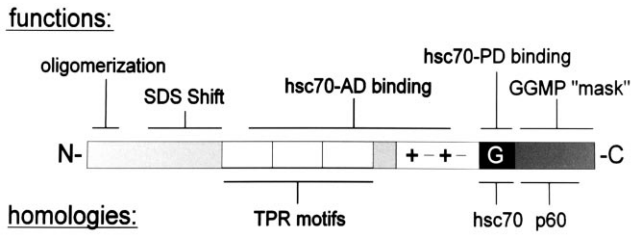

FIG. 5. Summary of Hip mutagenesis findings. Regions of human Hip required for various Hip interactions and behaviors are indicated above the linear diagram. For comparison, Hip regions containing motifs shared by other proteins are indicated below the diagram.

stained gel (Fig. 4, left panel) was dried and autoradiographed to detect associated Hip forms (right panel).

Correlating with hsp70-binding ability, mutants lacking either the TPR or highly charged regions were recovered at low to nonspecific binding levels in PR complexes (Fig. 4, lanes 3, 4, 7, and 8). N-303, which displays enhanced binding to hsp70 as an apparent misfolded substrate, was still able to enter PR complexes at levels comparable to that of WT Hip. Interestingly, mutant C-99, which like C-15 is deficient in homo-oligomerization activity, is recovered in PR complexes at near-WT levels. Still, since there are normal levels of endogenous Hip in the RL assembly mixes, it may be that one or more of the mutant Hip forms has the ability to enter PR complexes but lacks the ability to perform a subsequent function.

DISCUSSION

Normal hsp70 binding requires the TPR and highly charged regions. The results from Hip mutagenesis studies are summarized in Fig. 5. hsp70, which normally binds through its ATPase domain to Hip (3), was unable to bind Hip mutants lacking major portions of the TPR region (ΔTPR and N-148), and hsp70 binding was reduced in mutants lacking the adjacent highly charged region (Δcharge and N-226). hsp70 binding was retained in mutants with deletions outside the TPR and highly charged regions. From the present results, it cannot be concluded whether the charged region participates with the TPR region in directly binding to hsp70's ATPase domain or whether one region indirectly influences the other region's conformation. Another possibility is that the both the TPR and highly charged regions may not directly interact with hsp70's ATPase domain but may be required for interactions with DnaJ (3) or some other factor required to establish Hip-hsp70 complexes.

p60-homologous and GGMP repeat regions. Besides the mutants displaying loss of hsp70 binding, an additional hsp70-related phenotype was observed in the Hip mutant lacking the sequences homologous to p60 (N-303). Carboxy-terminal truncation of these sequences, which occur immediately downstream from the GGMP repeat region, resulted in enhanced hsp70 binding compared with that of WT Hip; however, unlike normal hsp70 binding to Hip, the additional hsp70 binding was insensitive to 0.4 M NaCl (Fig. 3A) and depended on sequences outside hsp70's ATPase domain (Fig. 3B). These data suggest that the salt-insensitive binding of full-length hsp70 to N-303 occurs through hsp70's C-terminal peptide-binding domain. A trivial explanation is that N-303 is aberrantly folded and is thus recognized by hsp70 as a misfolded substrate. Interestingly, though, the salt-insensitive binding is lost after a further truncation of the GGMP repeat region (N-276), and salt-insensitive hsp70 binding is minimal in all other Hip mutants. Thus, Hip's C terminus appears to function as a "mask" of the GGMP repeat region. It is intriguing that (i) Hip and p60 have homology within their C termini; (ii) p60 also binds hsp70, although not through sequences clearly homologous to Hip; and (iii) Hip and p60 appear concurrently during intermediate PR assembly stages (9, 12). Further experimentation may reveal a regulatable system by which Hip and p60 act in a concerted fashion to mediate hsp70 interactions with certain substrate proteins.

Unfortunately, the function of Hip's GGMP repeat was not made evident by deletion of this region (ΔGGMP). This motif is highly conserved, appearing as over 20 tandem repeats in a *Plasmodium* homolog of Hip (13). As noted previously (7), constitutively expressed hsp70 forms in vertebrates contain a short, degenerate GGMP repeat motif, whereas heat-shock-inducible forms lack this motif. As with Hip, though, no function has yet been demonstrated for the conserved GGMP region of hsc70.

The N-terminal region of Hip. Cross-linking analyses were used to examine Hip regions required for homo-oligomerization (Fig. 2A and B). The only mutants identified that failed to form cross-linked complexes were N-terminal truncation mutants. The first 14 amino acids are minimally required for oligomerization, since mutant C-15 failed to cross-link (Fig. 2B) and failed to copurify on Ni^{2+}-agarose with its His-tagged counterpart (Fig. 2C). Similar results were obtained with the more extensive N-terminal truncation mutants C-28, C-38, and C-99 (not all data presented). Since the C-terminal truncation mutant N-148 retained the ability to form cross-linked complexes (Fig. 2A), the initial 150 amino acids are sufficient to confer oligomerization.

Despite the loss of oligomerization ability, the N-terminal truncation mutant C-99 was still capable of binding hsp70 in a seemingly normal fashion (Fig. 3) and was able to enter PR complexes (Fig. 4). Perhaps homo-oligomerization is restricted to free Hip and is absent or unnecessary with hetero-oligomeric interactions involving hsp70. On the other hand, radiolabeled, monomeric Hip mutants such as C-99 may bind hsp70 and enter PR assembly complexes in a nonproductive manner.

The amino-terminal 100 amino acids of Hip divide into two clear regions on the basis of hydrophobicity plots. The first half of this region is weakly hydrophilic to hydrophobic, whereas the second half is strongly hydrophilic. As discussed above, the upstream, less hydrophilic region is more strongly implicated in oligomerization, but no potential motif in Hip's N-terminus that clearly suggests an oligomerization function has been identified.

A property of Hip other than oligomerization localizes to amino acids 50 to 100. It is apparent from the data summarized in Table 1 that WT Hip and several mutant forms migrate with anomalously high apparent molecular masses in SDS gels compared with predictions from their amino acid sequences. This behavior was observed for all Hip mutant forms except C-99, which lacks the highly hydrophilic region from amino acid 50 to 98. Mutant C-15 and mutants lacking the N-terminal 27 or 37 amino acids (not shown) retained slower gel migration, as did the C-terminal truncation mutant N-148, so the anomalous behavior probably resides in the region from amino acid 50 to 100. Posttranslational modifications within this region could possibly account for the altered mobility, but this does not seem likely. Each of the cDNAs has been expressed in bacteria, which are usually incapable of correct posttranslational modifications on eukaryotic proteins, and the recombinant products migrated identically to proteins expressed in vitro (not shown). Thus, it seems likely that amino acids 50 to 100 contain an

unusual inherent structure that, in the denatured protein, prevents full SDS association or otherwise retards Hip's gel mobility.

Potential functional impairment of Hip mutants. As discussed above, several Hip mutants displayed interesting phenotypes, but other than those deficient in hsp70-binding ability, all were recovered to some extent in PR complexes (Fig. 4 and results not shown). The coincident absence of hsp70 binding and PR association in Hip mutants ΔTPR, Δcharge, N-226, and N-148 supports a model in which Hip enters PR complexes indirectly through association with hsp70.

The simple presence of Hip mutants in PR complexes does not exclude the possibility that these forms are deficient in functions related to PR assembly. Hip forms retaining the ability to bind hsp70 but lacking other functions might associate unproductively with hsp70 and PR complexes. As discussed above, the observation that the GGMP repeat region serves as a good substrate for hsp70 binding in mutants lacking Hip's C terminus suggests potential regulatory roles for Hip in displacing hsp70 substrates or in PR assembly transitions involving p60. In the PR association assays (Fig. 4) there was a high background level of endogenous rabbit Hip that could dynamically compensate for less abundant Hip mutants with potential functional deficiencies. While Hip's role in hsp70-mediated processes such as PR assembly remains unresolved, efforts to exploit cell-free PR assembly as a model for defining Hip's function by using the mutants developed here and through the development of more refined Hip mutants are under way.

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