Functional Comparison of Human and *Drosophila* Hop Reveals Novel Role in Steroid Receptor Maturation*

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Hsp70/Hsp90 organizing protein (Hop) coordinates Hsp70 and Hsp90 interactions during assembly of steroid receptor complexes. Hop is composed of three tetra-tricopeptide repeat (TPR) domains (TPR1, TPR2a, and TPR2b) and two DP repeat domains (DP1 and DP2); Hsp70 interacts directly with TPR1 and Hsp90 with TPR2a, but the function of other domains is less clear. Human Hop and the *Saccharomyces cerevisiae* ortholog Sti1p, which share a common domain arrangement, are functionally interchangeable in a yeast growth assay and in supporting the efficient maturation of glucocorticoid receptor (GR) function. To gain a better understanding of Hop structure/function relationships, we have extended comparisons to the Hop ortholog from *Drosophila melanogaster* (dHop), which lacks DP1. Although dHop binds Hsp70 and Hsp90 and can rescue the growth defect in yeast lacking Sti1p, dHop failed to support GR function in yeast, which suggests a novel role for Hop in GR maturation that goes beyond Hsp binding. Chimeric Hop constructs combining human and *Drosophila* domains demonstrate that the C-terminal domain DP2 is critical for this previously unrecognized role in steroid receptor function.

Functional maturation of steroid receptors requires multistep assembly with molecular chaperones (1), and Hop, which binds both Hsp70 and Hsp90, can facilitate the progression through the intermediate stages of assembly (2). The early steps in assembling receptor/chaperone complexes are the binding of Hsp40 to a receptor monomer (3) followed by recruitment of Hsp70. Hop-Hsp90 complexes are then recruited to bind the receptor-associated Hsp70. In a transition that remains poorly understood, Hsp90 becomes directly associated with the receptor, and Hsp70 and Hop leave the complex. The Hsp90 co-chaperone p23 binds to Hsp90, stabilizing its association with the receptor, and immunophilin-related co-chaperones bind Hsp90 at a site vacated by Hop. Only when the receptor has achieved assembly with Hsp90 and p23 does it attain full hormone binding ability (4, 5).

Through its ability to simultaneously bind both Hsp70 and Hsp90, Hop serves as an adaptor to coordinate the recruitment of Hsp90 to intermediate receptor complexes containing Hsp70 (6). Hop can also inhibit Hsp90 ATPase, but it acts somewhat differently toward yeast or vertebrate Hsp90. Yeast Hsp90 has a much higher basal ATPase activity, and Sti1p, the Hop ortholog in *Saccharomyces cerevisiae* (7), inhibits this activity (8, 9). In contrast, human Hsp90 has very low basal ATPase activity that can be stimulated by binding to substrate, and Hop is able to inhibit the client-stimulated ATPase (10). In some settings Hop can also affect Hsp70 ATPase. For example, Sti1p dramatically stimulates activity of the yeast Ssa family of Hsp70 proteins (11), although Hop was not observed to stimulate the ATPase activity of vertebrate Hsp70 (12). Unlike most other receptor-associated chaperone components, Hop does not possess independent chaperone activity as determined by *in vitro* refolding assays (13, 14), so it is unlikely to act directly on a receptor.

In addition to cell-free assembly studies, yeast models have been used to show that deletion of Sti1p inhibits steroid receptor function (15). Yeast lacking functional Sti1p are viable but have retarded growth at elevated temperature or in the presence of minimal media (7). We have shown previously (6) that Sti1p can functionally replace human Hop (hHop)† using an *in vitro* receptor assembly system. We have also shown (16) that hHop can fully rescue the growth phenotype in a yeast strain (sti1Δ0) lacking the gene for Sti1p and that hHop can fully restore steroid receptor function in the sti1Δ0 background. Thus, it appears that Sti1p and hHop are functionally interchangeable.

Sti1p and hHop share over 50% amino acid sequence similarity and have a common domain structure consisting of three tetra-tricopeptide repeat domains (TPR1, TPR2a, and TPR2b) and two small domains (DP1 and DP2) containing a characteristic DP repeat motif (17). The domain arrangement is TPR1-DP1-TPR2a-TPR2b-DP2. Two of the Sti1p TPR domains have been individually crystallized (18) and have overall folds similar to each other and to the TPR domains from several other Hsp90-binding co-chaperones (reviewed in Ref. 19). Several lines of evidence suggest that the highly conserved EEVD sequence that terminates many eukaryotic Hsp90 and Hsp70 proteins directly interacts with a TPR binding pocket in co-chaperones.

Hop TPR2a is required for Hsp90 binding (20, 21); the Hsp90-specific peptide MEVVD co-crystallizes with TPR2a (18), and site-directed mutation of the EEVD terminus of Hsp90 is sufficient to disrupt binding with Hop (22–24). Therefore, EEVD-TPR interaction is critical for Hsp90 binding to Hop. Hop TPR1 is required for Hsp70 binding (20, 21), and the Hsp70-specific peptide PTIEEVD co-crystallizes with TPR1

† The abbreviations used are: hHop, human Hop; dHop, *Drosophila* Hop; TPR, tetra-tricopeptide repeat; RL, reticulocyte lysate; PR, progesterone receptor; GR, glucocorticoid receptor; IP, immunoprecipitation; WT, wild type.

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(18). On the other hand, deletion of PTTEEVD and up to 40 additional amino acids from the C terminus of Hsp70 do not prevent binding of truncated Hsp70 to Hop (16). In contrast to Hsp90, therefore, there must be one or more additional interaction modes between Hop and Hsp70, although the EEVD-TPR1 interaction is probably functionally important.

Based on analysis of Hop co-crystal structures (18), positively charged amino acids in the TPR binding pocket form a carboxylate clamp that interacts with the negatively charged EEVD. Point mutations of carboxylate clamp residues have been shown to disrupt binding of TPR-containing proteins to Hsp90 (25, 26) or Hsp70 (27). However, we also observed that carboxylate clamp mutations in Hop TPR domains had complex, unexpected effects on Hsp70 binding (16). We have further observed that mutation of the C-terminal DP2 domain of Hop disrupts Hsp70 binding (6). Our working model is that multiple Hop domains contribute to Hsp binding and that domain-domain interactions within Hop are likely important.

To further characterize the role of individual Hop domains in Hsp binding and in functional maturation of steroid receptors, we have undertaken a functional comparison between hHop and a Hop ortholog from Drosophila melanogaster (dHop), which shares ~65% sequence similarity. One unique feature of dHop is the absence of DP1, a domain of poorly understood function found in hHop and Sti1p (16), but there are sequence dissimilarities in other domains as well. In a variety of functional assays presented here, dHop is found to be similar to hHop and Sti1p, yet dHop fails to support steroid receptor maturation in vivo, and the deficiency maps to DP2.

MATERIALS AND METHODS

Antibodies—The following mouse monoclonal antibodies were used in these studies: F5 (28), which recognizes vertebrate Hop; S22 (15), which is specific for yeast Sti1p; PR22 (29), which recognizes the progesterone receptor (PR); BuGR2 (Affinity Bioreagents, Golden, CO), which recognizes the glucocorticoid receptor (GR); and anti-L3, which reacts with a yeast ribosomal protein (30). Novel mouse monoclonal antibodies specific for hHop (F5), dHop (DH1), and Sti1p (S22) were preadsorbed to protein G-Sepharose (1 mg of antibody/ml of packed resin). For each IP reaction, yeast lysate containing 5 mg of protein was incubated with 10 mg of immunogen overnight at 4 °C. Resin-bound complexes were washed three times in 1 ml of wash buffer (20 mM Tris-HCl, pH 7.4, 50 mM KC1, 0.5% Tween 20), resuspended in 10 ml of 2× SDS-PAGE sample buffer, and separated by gel electrophoresis. For immunoblots of yeast protein, aliquots of lysate (3 mg of protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunostained for Sti1p, hHop, dHop, GR, and α-tubulin (32). Mouse monoclonal antibodies for the two fragments were designed to contain the complementary sequences that surrounded the desired fusion site. The resulting DNA products were gel-purified and used as megaprimers in a reaction with the appropriate 5′-3′ primers to generate the full-length chimeric cDNA. The final PCR product was ligated into pSPUTK (Stratagene) for in vitro expression or into a yeast expression vector.

In Vitro Protein Binding Assays—Radiolabeled Hop forms were generated by in vitro expression (TnT kit, Promega, Madison, WI) in the presence of [35S]methionine. A 5-μl aliquot of each synthesis mixture was separated by SDS-PAGE; following autoradiography, protein bands were quantified by densitometry (Fluor-S Multi-Imager, Bio-Rad, Hercules, CA). For each co-IP, molar equivalents of each radiolabeled protein were mixed with 5 ml of immunogen-bound resins for 1 h, the resin-bound complexes were washed and then resuspended in 1 ml of sample. Regression analysis of the linear portion of each data set yielded a best fit line (typically, R2 > 0.98) of the slope of which (1000) is the growth-normalized rate of β-galactosidase expression.

Preparation of Yeast Lysates—Yeast strains were cultivated in 4 liters of minimal medium at 30 °C. At an A600 of 1.0–1.5 units, cells were harvested and the cell pellet (~20 g) was resuspended in 20 ml of ice-cold breakage buffer (20 mM Tris, pH 7.5, 100 mM KC1, 5 mM MgCl2, 10 mM β-mercaptoethanol plus Complete Mini protease inhibitor mixture (Roche Applied Science)). After the addition of 0.5-mm glass beads, cells were disrupted by five 30-s pulses (2 min of cooling between pulses) in a BeadBeater (BioSpec Products, Bartlesville, OK) or until ~50% of the cells were broken as determined by phase contrast microscopy. The cell slurry was maintained in an ice/ethanol bath during the disruption process. After centrifugation at 100,000 × g for 1 h, the lysate protein concentration was 3–5 mg/ml as determined by Coomassie Plus protein assay (Pierce).

Co-immunoprecipitations and Immunoblots of Yeast Lysates—Mouse antibodies specific for hHop (F5), dHop (DH1), and Sti1p (S22) were preadsorbed to protein G-Sepharose (1 mg of antibody/ml of packed resin). For each IP reaction, yeast lysate containing 5 μg of protein was incubated with 10 μl of immunogen for 4 h at 4 °C. Resin-bound complexes were washed three times in 1 ml of wash buffer (20 mM Tris-HCl, pH 7.4, 50 mM KC1, 0.5% Tween 20), resuspended in 10 μl of 2× SDS-PAGE sample buffer, and separated by gel electrophoresis. For immunoblots of yeast protein, aliquots of lysate (3 μg of protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and immunostained for Sti1p, hHop, dHop, GR, and α-tubulin.

Construction of Hop Chimeras—Chimeric cDNAs combining human and Drosophila Hop cDNA sequences were constructed by PCR. Primers for the two fragments were designed to contain the complementary sequences that surrounded the desired fusion site. The resulting DNA products were gel-purified and used as megaprimers in a reaction with the appropriate 5′-3′ primers to generate the full-length chimeric cDNA. The final PCR product was ligated into pSPUTK (Stratagene) for in vitro expression or into a yeast expression vector.

Yeast Strains and Plasmids—All S. cerevisiae strains were generated in the W303α background (MATα leu2–112 ura3–1 trp1–1 his3–11, 15 ade2–1 can1–100 GAL SUCl2). Plasmids were introduced into wild type or mutant strains as described previously (31). Briefly, yeast strains were grown in selective media at 25 °C to an absorbance at 600 nm (A600) of 0.05–0.12 units. Growth was monitored by spectrophotometry for 30 min before hormone addition to ensure that the culture was in exponential phase. Deoxycorticosterone was added to the culture at a concentration of 50 nM final concentration. To assay for β-galactosidase activity 100 μl of culture was withdrawn and immediately added to 100 μl of the Gal-ScreenTM substrate (Applied Biosystems, Bedford, MA) in 96-well microtiter plates at room temperature. Five seconds was taken at 10-min intervals starting 70–80 min after hormone addition and read in a luminometer. To determine the rate of reporter expression, β-galactosidase induction curves were first generated by plotting relative light units against the A600 of the sample culture. Regression analysis of the linear portion of each data set produced a best fit line (typically, R2 > 0.98) of which (1000) is the growth-normalized rate of β-galactosidase expression.

FIG. 1. Rescue of sti1ΔO growth defect. 5-fold serial dilutions of WT or sti1ΔO yeast were spotted on minimal media plates and incubated at 30 or 37 °C for 5 days. The sti1ΔO strain was used without transformation or was transformed with a plasmid expressing either hHop or dHop as indicated on the left. Growth at 37 °C was retarded for sti1ΔO cells but not for WT or sti1ΔO cells expressing exogenous Hop.
RESULTS

Yeast that lack Sti1p grow normally at 30 °C but more slowly at 37 °C (7). We determined previously that hHop could functionally substitute for Sti1p as it restores normal growth at elevated temperature (16). To initiate an examination of dHop function, we tested whether dHop can rescue the temperature-sensitive growth phenotype of the sti1Δ yeast strain. As shown in Fig. 1, dHop and hHop both restore growth at 37 °C in a sti1Δ strain; thus, it appears that dHop is fully functional in this growth assay.

Further functional analysis of dHop was performed using the well established yeast model for steroid receptor function. The Yamamoto laboratory (35) first showed that yeast expressing a vertebrate steroid receptor contain the minimal cellular factors needed to support hormone-inducible activation of an appropriate reporter gene. The Lindquist laboratory (15) showed that Sti1p is necessary for full receptor activity in the yeast background, and we subsequently demonstrated (16) that hHop will fully rescue GR function in sti1Δ. Here we extend those studies by demonstrating the ability of dHop to restore GR function (Fig. 2). In sti1Δ transformed with empty vector, hormone-induced activity remains near the uninduced level, but wild-type activity is restored by expressing hHop. In contrast to the human protein, dHop was unable to restore GR function even though dHop can rescue the temperature-sensitive growth phenotype in sti1Δ. Western immunostains confirmed that Sti1p is absent in sti1Δ cells (Fig. 2B), that hHop and dHop are expressed in transformed yeast (Fig. 2C), and that the level of GR protein is similar in all strains (Fig. 2D).

To gain insight into the nature of the defect that prevents dHop support of GR function, we examined protein interactions of dHop. A critical feature of Hop-mediated assembly of receptor complexes is the binding of Hop to both Hsp70 and Hsp90. To examine whether dHop and hHop interact differently with Hsp70, Hsp90, or steroid receptor complexes, co-immunoprecipitation assays were performed in rabbit RL, a medium commonly used for cell-free assembly of receptor complexes. As seen in Fig. 3, radiolabeled hHop and dHop co-immunoprecipitated equally with either Hsp70 or Hsp90 (Fig. 3A). Furthermore, hHop and dHop seemed to assemble equally well in PR complexes (Fig. 3B). The co-IP analysis was extended to yeast extracts, as shown in Fig. 4. Sti1p, hHop, and dHop complexes were individually targeted using specific monoclonal antibodies. To control for nonspecific binding of chaperones to immune
complexes, IP was performed at low ionic strength, which preserves Hsp70-Hop-Hsp90 complexes, and at elevated ionic strength, which dissociates Hsp binding to Hop. Further controls included IP reactions using extracts from yeast lacking the targeted Hop forms. Yeast Hsp90 (Hsc82, confirmed by Western immunostaining) and Hsp70 forms (Ssa1 and Ssa2 as determined by mass spectrometry) were specifically recovered with each of the three Hop orthologs.

To better understand why dHop fails to support GR maturation, the deduced amino acid sequences for hHop, Sti1p, and dHop were aligned to identify regions of dissimilarity among the orthologs (Fig. 5). The most striking difference is the apparent absence of DP1 in dHop. Other domains are intact, although amino acid differences within these domains might be critical. As an experimental approach to map the relevant differences in hHop and dHop, a series of chimeric constructs was generated in which domains were exchanged between orthologs. The exchange boundaries are indicated by arrowheads in Fig. 5, and the resulting chimeras are illustrated in Fig. 6.

Radiolabeled wild-type and chimeric proteins were generated in vitro and tested for co-IP with Hsp70 (Fig. 7A), Hsp90 (Fig. 7B), and PR (Fig. 7C). In addition, wild-type and chimeric Hop forms were functionally compared after separately transforming the sti1ΔH0 GR reporter strain with an expression plasmid for each Hop form. Results for the 4 chimeras that generated reporter signals above background are plotted in Fig. 8. The results from all chimera assays are summarized in Fig. 9.

One of the clear findings is that transfer of the dHop C-terminal DP2 domain to hHop (chimera I) is sufficient to block Hop-mediated GR activation even though Hsp binding and receptor assembly in vitro are unaffected. Another potentially important observation is that placing the human DP1 domain into dHop (chimera C) completely disrupts Hsp binding. The converse hHop construct in which DP1 has been removed (chimera D) retains Hsp binding and GR activation.

**DISCUSSION**

A comparative approach has uncovered unique features of Hop function that relate to its role in GR maturation and function. The fact that dHop, similar to human and yeast orthologs, can support yeast growth at elevated temperatures, and yet dHop alone fails to support GR activity, provides a unique demonstration that Hop functions differently in these roles. The molecular targets of Hop that are relevant to temperature-sensitive growth have not been identified, but multiple clients of Hsp70 and Hsp90 could be adversely affected by the loss of Hop and collectively contribute to growth retardation. GR, however, must be affected by Hop in a distinct manner since dHop fails to support GR activity.

For Hop to support steroid receptor assembly and functional maturation it must bind both Hsp70 and Hsp90 (20). Because Hop does not directly interact with a receptor, it has been considered an adaptor protein that helps recruit Hsp90 to pre-existing Hsp70-receptor complexes (6). However, in light of our results with chimera I in which binding to Hsp70 and Hsp90 remains maximal, whereas recovery of GR function is minimal, there must be an additional Hop activity other than passive binding to Hsp70 and Hsp90 that is critical for GR function in vivo. This additional activity maps most closely with DP2.

We have shown that DP2 forms an independent domain that is readily disrupted by point mutations in the conserved DP repeat motif (36). A consequence of DP2 disruption is abrogate...
tion of Hsp70 binding, which provides evidence that DP2 and TPR1 interact in some manner to support Hsp70 binding ability. The DP2 domain of dHop retains DP repeat sites and shares greater sequence similarity to hHop (70% similarity) than does the DP2 domain of Sti1p (60% similarity). A key observation in this study is that exchanging DP2 between hHop and dHop does not grossly affect Hsp70 binding; nonetheless, chimera I (hHop with Drosophila DP2) fails to support GR activity, and the converse chimera J (dHop with human DP2) gains the ability to enhance GR activity. Therefore, DP2 contributes something other than Hsp70 binding to promote GR function.

It is intriguing that Hop has a second DP-related domain, DP1, inserted between TPR1 and TPR2a, and that DP1 is absent from dHop. DP1 does not appear to be critical for hHop function because mutations in the DP repeat motif had no effect on Hsp binding or GR activity (16), and deletion of DP1 from hHop (chimera D) only partially reduced Hsp binding and GR activity. On the other hand, insertion of human DP1 into Drosophila Hop and Steroid Receptor Function

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