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SHORT COMMUNICATION

Single-point mutation in a conserved TPR domain of Hip disrupts enhancement of glucocorticoid receptor signaling

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Abstract The Hsp70-interacting protein Hip has been identified as a transient participant in the assembly of both glucocorticoid (GR) and progesterone receptor complexes. Although it has been difficult to identify a physiological role for Hip, it is believed to have intrinsic chaperoning properties and has been identified as a potential anti-apoptotic target of Granzyme B. In vitro assays have provided evidence that Hip may interact with GR complexes in an Hsp70 independent manner and can enhance the function of GR in hormone based reporter assays. In this study, a cDNA for human Hip was used in mutational analysis to map Hip function to critical structural elements. A single amino acid substitution (L211S) resulted in a loss of Hip function. This mutation also appears to disrupt the interaction of Hip with Hsp70 in vitro. Failure to recover Hip-L211S constructs in co-immunoprecipitation assays with an Hsp70 monoclonal antibody suggests that the mutation is unlikely to result in a misfolded substrate.

Keywords Hip · Hsp70 · Glucocorticoid receptor · Molecular chaperone

Introduction

Steroid receptors are hormone-dependent transcription factors that are typically found in heteromeric complexes with Hsp90 and other chaperones prior to hormone binding. In vitro assembly studies have revealed an ordered pathway of chaperone assembly with steroid receptors that is required to establish and maintain the hormone binding ability of several steroid receptors. Hsp90, Hsp70, Hsp40, Hop, and p23 are minimally required for efficient functional maturation of glucocorticoid receptor (GR) (reviewed in Pratt and Toft 2003), but several additional co-chaperones participate in an assembly of native receptor complexes. Hip, an Hsp70 binding co-chaperone, stabilizes the ADP bound state of Hsp70 and promotes Hsp70 binding to substrate proteins (Höhfeld et al. 1995). In addition, Hip is known to transiently enter the steroid receptor complex at an intermediate assembly stage and was first observed as a component of progesterone receptor (PR) complexes (Smith 1993). In an attempt to address the potential physiological role of Hip, Kanelakis et al. (2000) overexpressed Hip in a mammalian cell model but reported little effect on GR function. These data, however, may simply reflect that endogenous Hip levels are above the rate-limiting level for GR maturation. Hip overexpression is able to reverse inhibition of GR function caused by overexpression of BAG1, an Hsp70 co-chaperone that inhibits Hsp70 binding to substrates (Takayama et al. 1997), but this phenomenon does not exclude the possibility that Hip plays an active role in GR maturation apart from countering BAG1 actions.

Excluding a potential physiological role for Hip in vertebrate cells has been problematic since these cells typically express Hip at relatively high levels and also express multiple BAG1 isoforms. As an alternative to vertebrate cells, the yeast *Saccharomyces cerevisiae*, which lacks a homologous Hip gene as well as a clear ortholog for cytoplasmic BAG1, provides a unique cellular background in which Hip can be introduced de novo and interactions with BAG1 excluded. Yeast also lack steroid receptor genes; nonetheless, Yamamoto and colleagues demonstrated that a glucocorticoid-responsive system could be generated by

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transforming yeast with plasmids expressing GR and a reporter gene under control of glucocorticoid response elements (Schena and Yamamoto 1988). The yeast model for steroid signaling has been widely exploited, especially by investigators exploring the function of receptor-associated chaperones. Although GR will function in a hormone-dependent manner in yeast, indicating that minimal chaperone and transcriptional factors required for function are present, GR function is not maximal. Nelson et al. (2004) previously observed that introduction of human Hip into *S. cerevisiae* can double hormone-dependent GR transactivation of reporter plasmids. This enhancement of hormone-dependent expression is not observed for other nuclear receptors and appears to be specific for GR activation. Hip, however, does not appear to affect functional maturation of GR hormone binding ability in vitro. Resolution of these opposing data about the importance of Hip in steroid receptor function may lie in experiments that elucidate other potential cellular roles for Hip in steroid receptor signaling independent of Hsp70 binding. This study describes a unique approach utilizing yeast genetics to understand what features of Hip are required for enhancement of GR function.

Results

This study took advantage of the yeast model to genetically screen for Hip mutants that fail to enhance GR function in a first step towards understanding amino acid contacts critical to Hip function. Hip consists of an N-terminal oligomerization domain, a central tetratricopeptide repeat domain and adjacent highly charged region, both of which are required for Hsp70 binding, and a C-terminal DP-repeat domain that influences Hip release from Hsp70 (Fig. 1a). A library of random Hip mutants was generated by low-fidelity polymerase chain reaction (PCR) of Hip cDNA and transformed into a yeast strain expressing the *CAN1* gene in a hormone- and GR-dependent manner. Yeast were grown in the presence of canavanine and deoxycorticosterone (DOC) to select for colonies that express low levels of the *CAN1* gene and are thus canavanine-resistant. Resistant colonies were further screened by Western immunostaining of cell extracts to verify expression of full-length Hip. A large array of mutant Hip cDNAs were recovered that represented missense mutations in over one third of Hip codons (Fig. 1b). To verify defects in Hip function, mutant Hip forms were transformed into a separate GR reporter expression strain and compared with wild-type (wt) Hip for ability to enhance GR function. Single-point mutants were generated from selected Hip mutants containing more than one missense mutation and were functionally characterized in vitro for Hsp70-binding properties (Fig. 1b).

Functional Hip alters yeast growth in the presence of canavanine

In order to identify mutations resulting in loss of Hip function the growth curve of Hip mutants and the hormone-dependent GR transactivation of reporter plasmids in yeast cells containing an integrated *CAN1* gene driven by a hormone responsive element were examined. In the presence of 50 μg/ml canavanine and 50 nM DOC, yeast transformed with GR only, or GR and wt human Hip grew significantly slower than yeast transformed with a mutant Hip that has lost the ability to enhance the hormone-

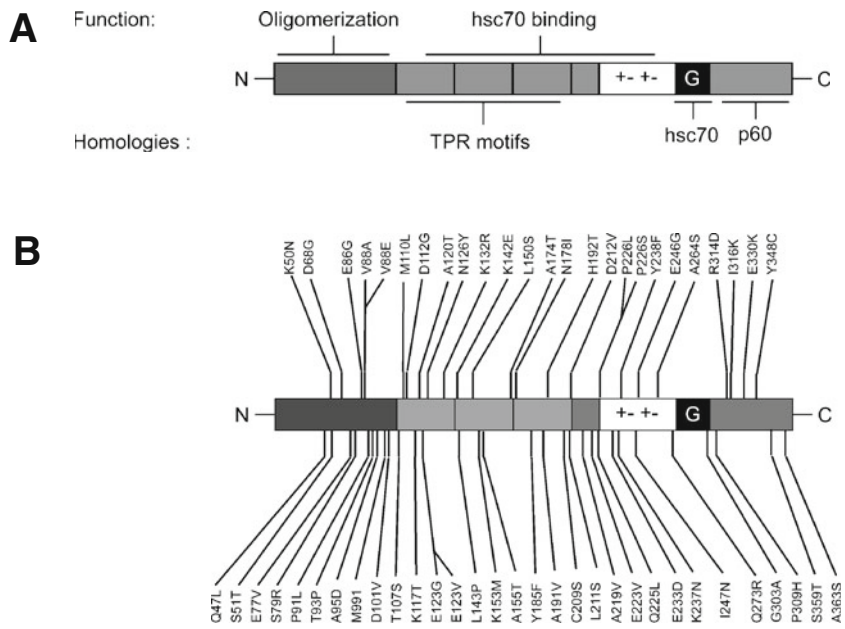

Fig. 1 **a** Functional domains of Hip targeted for mutagenesis are indicated above the diagram with known regions of homology with other protein motifs indicated below the diagram. **b** Mapping of point mutations created during error prone PCR to the functional domains of wt Hip that were screened for loss of function

dependent GR transactivation of the reporter plasmid (Fig. 2a) These results indicate growth under these conditions is sufficient to allow the differentiation of colonies expressing wt Hip and those expressing Hip mutants with a loss or significant decrease in function.

Identifying loss of function mutants generated by random mutagenesis

Yeast colonies displaying a fast growth phenotype in the presence of canavanine and DOC were further assayed for hormone-dependent GR transactivation of a β-galactosidase (β-Gal) reporter plasmid. Those mutants exhibiting a significant decrease in expression of the reporter plasmid in comparison to yeast transformed with wt Hip were re-plated on selective media without canavanine or DOC. Figure 2b shows a representative graph of the activity of Hip mutants identified through this initial screen. PCR of the full-length cDNA was then performed to verify the Hip plasmid was still present and the resulting amplimer was sequenced and point mutations were mapped to specific domains within the Hip protein (Fig. 1b). Error prone PCR of wt Hip consistently generated mutants containing two to four missense mutations that were not localized to any specific domain of the Hip protein (Fig. 1b). In all, 123 mutants were analyzed, and of those, 13 (11%) were determined to contain early termination codons and 1 (<1%) resulted in a frame-shift mutation.

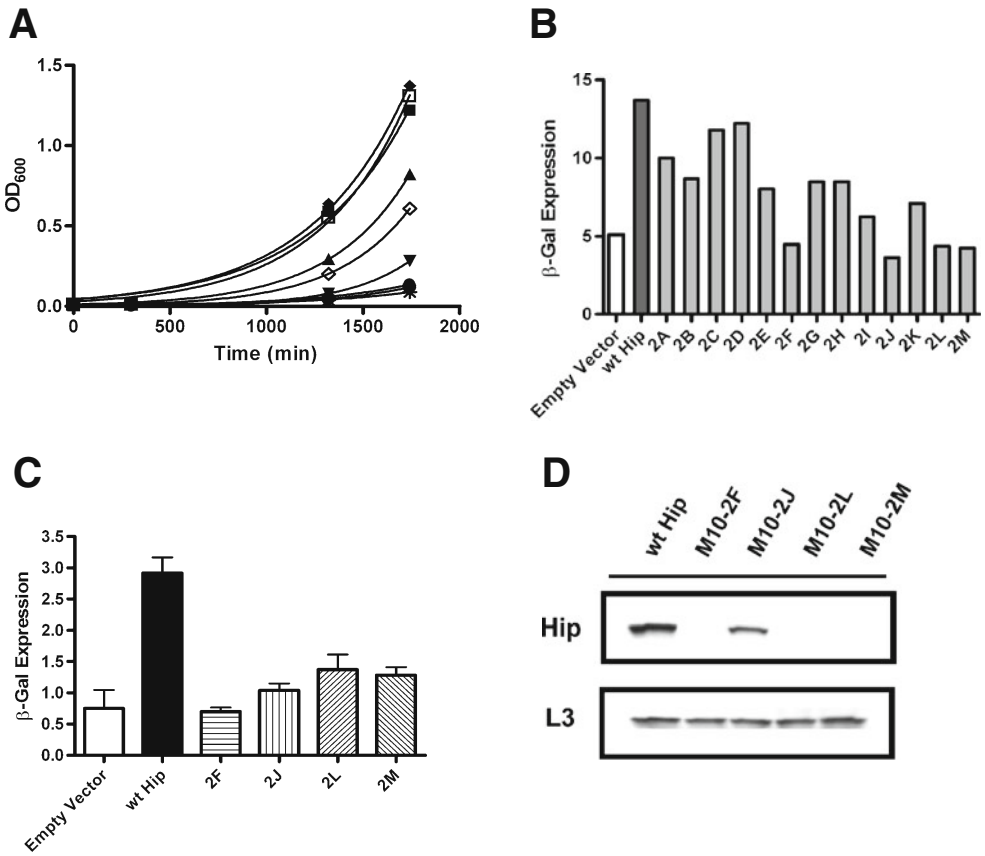

Fig. 2 **a** Growth curves at 30°C were determined in the presence of 50 μg/mlL-canavine sulfate salt (Sigma) for wild-type (*wt*) Hip (*asterisk*), empty vector (*filled diamonds*), GR alone (*empty diamonds*), or a library of random Hip mutants generated by low-fidelity PCR of wt Hip cDNA and GR (all others) and co-expressed in YNK512 plasmids generated via homologous recombination. **b** Colonies displaying a fast growth phenotype in the presence of canavanine were assayed for enhancement of GR function using the hormone-dependent reporter assay as previously reported (Nelson et al. 2004). **c** For mutants that showed defects in the enhancement of GR signaling, mutant Hip cDNAs were isolated from colony PCR reactions and ligated to the yeast expression vector p423GPD and transformed into a separate GR reporter expression strain (DSY-1000) to verify this effect was due to defects in Hip function. Five independent transformants were compared with wt Hip for ability to enhance GR function. To determine the rate of reporter expression, β-galactosidase induction curves were first generated by plotting relative light units against the OD_{600} of the culture sample. Regression analysis of this linear portion of each data set yielded a best-fit line (typically, $R^2>0.98$) the slope of which is the growth rate-normalized rate of β-galactosidase expression. For convenience, the reporter expression units are defined as the slope/100. **d** Western immunoblots —whole-cell extracts were fractionated by SDS-PAGE and transferred to polyvinylidene fluoride membrane for immunoblot analysis using the mouse monoclonal antibody 2 G6 (anti-Hip; 1:5,000 dilution) to verify the expression of full-length Hip constructs in yeast isolates displaying a loss of function

Western analysis was performed to verify the Hip construct was expressed and stable within the yeast colonies selected. Colonies no longer containing the Hip plasmid or containing early termination codons were excluded from further analysis.

Verifying loss of function

To verify the loss of function was due to the mutations in the Hip protein and not a secondary problem within the yeast colony, mutant Hip cDNA was co-transformed with a gapped yeast vector into a secondary yeast strain (DSY-1100) and assayed the hormone-dependent GR transactivation of a β-Gal reporter plasmid for five independent isolates and compared them with the enhancement of reporter expression exhibited by wt Hip. Four isolated mutants, Hip-2 F, 2 J, 2 L, and 2 M showed a significant loss of function (Fig. 2c, unpaired *t* test, $df=4$; $p<0.05$). Table 1 provides a list of point mutations contained within Hip mutants 2 F, 2 J, 2 L, and 2 M. Of those four, only Hip-2 J showed stable expression of full-length Hip after Western analysis of protein extracts isolated from each yeast colony (Fig. 2d).

Analysis of individual point mutations within Hip

The point mutants L211S, P226S, and G303A were produced by site-directed mutagenesis of wt Hip and transformed into yeast strain DSY-1100. β-Gal expression assays were performed on five independent isolates of DSY-1100 transformed with wt Hip, Hip-2J, Hip L211S, Hip P226S, or Hip G303A. Figure 3a shows the point mutant L211S displays significantly lower enhancement of hormone-dependent β-gal expression (unpaired *t* test, $df=4$; $p<0.05$). Although the point mutant P226S appears to have a small effect on Hip's enhancement of GR transactivation, it was not statistically significant (Fig. 3a). Hip G303A appears to have no affect on Hip function

Table 1 Multiple point mutations contained within the Hip mutants with confirmed loss function in hormone-dependent reporter assay

Hip-2 F	T107S
	M110L
	Q225L
Hip-2 J	L211S
	P226S
	G303A
Hip-2 L	L143P
	N178I
	A363S
Hip-2 M	D72Y
	L81Q
	A156D
	A324V

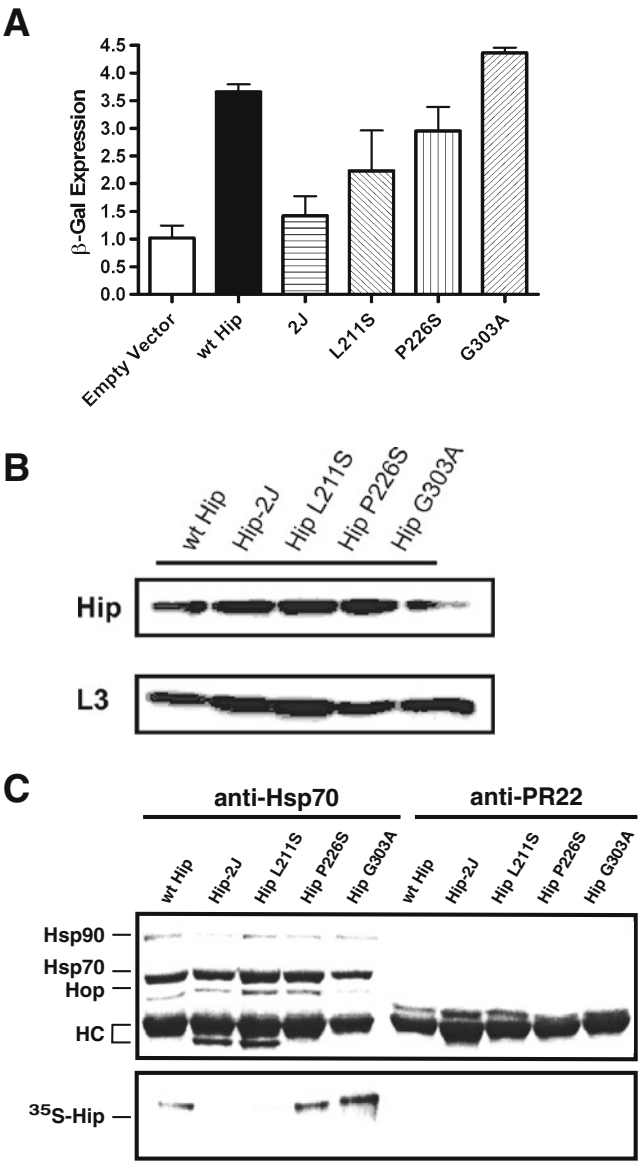

Fig. 3 **a** Hormone reporter assay—β-galactosidase expression was used to assess the impact of individual point mutations (*lined bars*) on the ability of wt Hip (*black bar*) or empty vector (*open bar*) to enhance GR function. **b** Western immunoblots—whole-cell extracts were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane for immunoblot analysis using the mouse monoclonal antibody 2 G6 (anti-Hip; 1:5,000 dilution) to verify the expression of full-length Hip constructs in yeast transformed with Hip point mutants. **c** In vitro co-immunoprecipitation—the abilities of Hip point mutants to bind Hsp70 were assessed utilizing Hsp70 immobilized on an immunoaffinity resin. Anti-Hsp70 monoclonal antibody BB70 was adsorbed to protein G-Sepharose (Pharmacia Biotech, Piscataway, NJ) and used to immunoprecipitate Hsp70 from rabbit RL (1:1; from Green Hectares, Oregon, WI) that was supplemented with a radiolabeled Hip form. Each sample contained the same molar equivalent of radiolabeled Hip or Hip mutant in 100 μl RL with 10 μg BB70 on a 15-μl resin pellet. Proteins adsorbed to resin were separated by SDS-PAGE and visualized by Coomassie blue staining and autoradiography of the dried gel. Control antibody used to compare background binding for the Hsp70 forms was the anti-PR monoclonal antibody, PR22

(Fig. 3a). Western analysis of protein extracts indicates each Hip construct is expressed in equivalent amounts and therefore the differences in enhancement of GR transactivation are not due to the level or stability of a particular Hip construct (Fig. 3b). Kruskal–Wallis one-way analysis of variance on ranks indicated no significant difference in the relative level of protein expression across constructs (H=1.063; df=4; p=0.900).

Hip L211S is defective in Hsp70 binding

To determine if the loss of function identified in the Hip mutant L211S was related to a defect in the ability of Hip L211S to associate with Hsp70, co-immunoprecipitation assays with an Hsp70 monoclonal antibody were performed with each Hip construct. Figure 3c shows neither the triple mutant Hip-2 J nor the point mutant Hip L211S are co-precipitated with Hsp70. The mutation P226S apparently has little effect on Hsp70 binding and the G303A mutation may even result in an increase in affinity for Hsp70 (Fig. 3c). Co-immunoprecipitation of the Hip constructs with a control antibody, PR-22, verifies that S^{35}-labeled Hip constructs pulled down by co-IP are specifically bound to Hsp70 (Fig. 3c).

Discussion

For several years Hip has been recognized as a transient component of native steroid receptor complexes, yet evidence is limited that this transient association is physiologically relevant. Hip is known to counteract the inhibitory actions of BAG1 with respect to GR assembly in a minimal system, but it is not known if this is a specific cellular function of Hip or if it simply reflects competitive displacement of BAG1 by Hip from Hsp70 complexes in vitro. Interestingly, although the minimal assembly system will restore GR hormone binding ability, more recent studies indicate GR assembled in a minimal system fails to activate transcription in a cell-free assay (Thackray et al. 2003). Yet, upon addition of rabbit reticulocyte lysate (RL) to the system, hormone-dependent transcriptional activation is re-established. These studies indicate one or more unknown components of RL are required for complete activation of GR. It is possible that Hip is a missing factor provided by RL. Earlier studies attempting to verify this possibility through immunodepletion of Hip from RL were unsuccessful in producing defects in receptor assembly complexes, due perhaps to residual Hip protein (Prapapanich et al. 1998). In 2004, Nelson et al. provided support for a potential physiological role of Hip by showing a doubling of hormone-dependent reporter gene expression. In an effort to identify potential amino acid residues important for the apparent increase in GR efficiency previously reported (Nelson et al. 2004), a library of random Hip mutants was screened in a yeast model to identify colonies displaying a significant reduction in Hip-mediated enhancement of GR activation. Although yeast cells do not perfectly mirror the physiology of mammalian cells, this approach has significant advantages over traditional strategies employed to examine Hip function in mammalian cells. RNAi-mediated knockdown of Hip is feasible; however, a considerable drawback to this approach is the inability of this approach to discriminate between BAG1-dependent and BAG1-independent functions of Hip. Due to the multiple isoforms of BAG1 expressed in mammalian cells, it is not practical to attempt simultaneous knockdown of BAG1 activity to generate an environment for the independent function of Hip. For this reason, the yeast model is a particularly attractive alternative since *S. cerevisiae* do not contain Hip-related or BAG1-related genes that would be likely to participate in steroid receptor assembly. Although deletion of entire Hip domains have been previously shown to disrupt this function (Nelson et al. 2004), this is the first report to identify changes to a single amino acid that results in a similar functional loss.

Potential mechanisms for Hip function

The mechanism by which Hip promotes functional maturation of GR is unknown, but several proposed mechanisms can be excluded. Historically, Hip was believed to function in GR assembly through interactions with another co-chaperone, Hop, through a common complex with Hsp70. Hip and Hop appear simultaneously at intermediate stages of steroid receptor assembly and both bind Hsp70, so it is reasonable to speculate that these co-chaperones function in a cooperative manner. The Hop ortholog in yeast, Sti1p, is required for GR maturation and Hip can partially rescue GR defects in a Sti1p deficient strain, further supporting a shared role for Hip and Hop in GR maturation (Chang et al. 1997; Nelson et al. 2004). Multiple sequence alignment of several members of the family of proteins containing TPR domains, including the TPR2a domain of Hop, indicates the L211S mutation occurs within the final turn of the last helix associated with the TPR motif. Without crystal structure information for the Hip protein, it is not realistic to speculate on the effect this mutation is having on Hsp70 binding and how this could translate to a change in the enhancement of GR activity. Although it would seem the inability to bind Hsp70 prevents Hip from entering the heterocomplex and thus is unable to affect the efficiency of GR maturation, previous studies have indicated Hip does not require Hsp70 binding to function in GR assembly. Nelson et al. (2004) reported deletion of the TPR domain eliminates Hsp70 binding yet Hip retains its activity in GR reporter assays. In addition co-expression of rat Hsp70 with

Hip inhibited rather than enhanced Hip function (Nelson et al. 2004). In addition, Hop containing a point mutation that disrupts Hsp70 binding will also partially rescue GR function in the Sti1p deficient strain (Carrigan et al. 2005). Therefore, Hip and Hop may both have Hsp70-independent roles in GR maturation. While Hip's role in hsp70-mediated processes such as GR assembly remains unresolved, efforts to exploit cell-free GR assembly as a model for defining Hip's function by using the mutants developed here and through the development of more refined Hip mutants show potential for eventual elucidation of the physiological role of Hip's transient association with steroid receptor complexes.

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