Functional Coevolutionary Networks of the Hsp70–Hop–Hsp90 System Revealed through Computational Analyses

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Currently, the identification of groups of amino acid residues that are important in the function, structure, or interaction of a protein can be both costly and prohibitively complex, involving vast numbers of mutagenesis experiments. Here, we present the application of a novel computational method, which identifies the presence of coevolution in a data set, thereby enabling the a priori identification of amino acid residues that play an important role in protein function. We have applied this method to the heat shock protein (Hsp) protein-folding system, studying the network between Hsp70, Hsp90, and Hop (heat shock–organizing protein). Our analysis has identified functional residues within the tetratricopeptide repeat (TPR) 1 and 2A domains in Hop, previously shown to be interacting with Hsp70 and Hsp90, respectively. Further, we have identified significant residues elsewhere in Hop within domains that have been recently proposed as being important for Hop interaction with Hsp70 and/or Hsp90. In addition, several amino acid sites present in groups of coevolution were identified as 3-dimensionally or linearly proximal to functionally important sites or domains. Based on our results, we also investigate a further functional domain within Hop, between TPR1 and TPR2A, which we suggest as being functionally important in the interaction of Hop with both Hsp70 and Hsp90 whether directly or otherwise. Our method has identified all the previously characterized functionally important regions in this system, thereby indicating the power of this method in the a priori identification of important regions for site-directed mutagenesis studies.

Introduction

Uncovering the functional and structural properties of protein residues is challenging both theoretically and experimentally. As a way to identify functional amino acid sites, researchers have investigated the mutational dynamics of residues by the development of mathematical models able to describe the evolution of these amino acid sites throughout millions of years. Accurate description of these mutational dynamics has been, however, halted by the simplicity of the mathematical models proposed so far. As an alternative to this approach, researchers have emulated the evolution of sequences by mutagenesis experiments in genes extracted from single organisms. Unfortunately, this approach can prove both costly and prohibitively slow mainly due to the multifactorial evolutionary and functional nature of amino acid sites, which relies on the coevolutionary relationships between protein functional domains (Fares 2006). Development of predictive computational tools to identify a priori the importance of specific amino acid sites in a protein is instrumental to substantially reduce experimental efforts to identify important protein functional regions.

The advent of increased computing power and the availability of full-genome sequences from a myriad of organisms have enabled the development of numerous methods for the in silico analysis of proteins. This has been paralleled by an increase in the sensitivity of methods supported by complex and more realistic mathematical models. We have recently described a method, Coevolution Analysis using Protein Sequences (CAPS), to identify intraprotein coevolution (Fares and Travers 2006). Even though coevolution is not synonymous with protein–protein interaction (Pritchard and Dufton 2000; Suel et al. 2003; Gloor et al. 2005), identifying coevolutionary events can highlight structural, functional, or interaction dependencies between 2 proteins. Coevolution has been studied, with varying degrees of sensitivity, in heat shock protein 90 (Hsp90), GroEL, the HIV *gag* and *env* genes, tetrapod myoglobin sequences, as well as with 846 rat–human–dog trios for which protein sequences were available (Korber et al. 1993; Pollock et al. 1999; Shim Choi et al. 2005; Fares and Travers 2006). Describing protein–protein functional relationships through coevolutionary analyses can help in the identification of the main genetic factors responsible for the robustness of biological systems. Essential cell cycles, such as the ATPase cycle of Hsp90, present strong functional dependencies between their protein components. The identification of these dependencies can aid in the identification of the important factors governing this vital cell cycle. However, most efforts have been devoted to the identification of protein–protein interactions and little attention has been put on the identification of functional coevolution between proteins. To illustrate the importance of establishing coevolutionary functional relationships between proteins, we completed the development of our previously published method (CAPS; Fares and Travers 2006) for the detection of interprotein evolutionary dependencies in the ATPase cycle of Hsp90. This cycle is essential for cell viability, and yet, the evolutionary and functional dependencies between its protein components remain obscure.

Hsp play an essential role in cell viability at normal physiological conditions by protecting their cellular protein clients from degradation and misfolding (Hightower 1991). Hsp90 is a specialized chaperone involved in the folding and maturation of key regulatory proteins, such as steroid hormone receptors, transcription factors, and kinases (Wegele et al. 2004; Atchley et al. 2005). Most of the slow-folding client-regulatory proteins of Hsp70 chaperones require further processing by the Hsp90 machinery. Both Hsp70 and Hsp90 chaperones are ATP dependent, and, in both chaperones, ATP hydrolysis results in conformational changes that are responsible for the folding and activation of the

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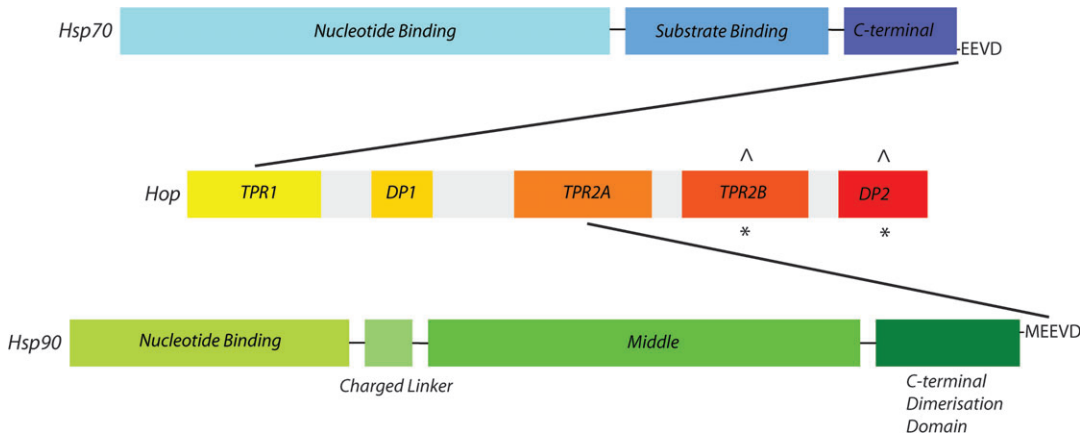

FIG. 1.—Overview of the domains of Hsp70, Hsp90, and Hop. Previously determined interactions between Hop and Hsp70/Hsp90 are shown with a solid line. Regions in Hop suggested to be involved in binding of hsp70 (^) or Hsp90 (*) are also shown.

client proteins (Prodromou et al. 2000; Richter et al. 2001; Shomura et al. 2005).

Generally, Hsp70 consists of a 44-kDa N-terminal nucleotide-binding domain (NBD), an 18-kDa substrate-binding domain (SBD), and a 10-kDa C-terminal domain (Flaherty et al. 1990; Zhu et al. 1996). Three-dimensional structures have been obtained for the Hsp70 NBD (Flaherty et al. 1990; Harrison et al. 1997; Osipiuk et al. 1999) and the SBD (Zhu et al. 1996), and more recently, the NBD and SBD (amino acids 1–501) of *Thermus thermophilus* DnaK was resolved (Revington et al. 2005) as was a functionally intact crystal structure of bovine Hsc70 (Jiang et al. 2005). Hsp90 exists as a dimer and consists of an N-terminal NBD linked by a flexible charged linker to the middle domain and a C-terminal dimerization domain. Three-dimensional structures have been resolved for the Hsp90 NBD (Prodromou, Roe, O'Brien et al. 1997; Stebbins et al. 1997) and middle domains (Meyer et al. 2003). Recently, in a rigorous study the 3-dimensional structure of the full Hsp90 bound to the cochaperone p23 has also been resolved, and the main conformational changes resulted from this binding have been precisely and elegantly detailed (Ali et al. 2006).

Cochaperones are an integral part of the chaperone protein–folding mechanisms and are involved in regulation of chaperone activity. They also present client proteins to chaperones and enable the cooperative relationship between different chaperone systems. A minimum system of Hsp40, Hsp70, Hsp90, and the cochaperones Hop (heat shock–organizing protein, Sti1 in yeast) and p23 (Sba1 in yeast) has been observed to be sufficient for the assembly of steroid hormone receptor complexes (Dittmar et al. 1998; Kosano et al. 1998). The definitive function of p23 is as yet unknown, although it is known that p23 binds to and stabilizes the ATP-bound form of Hsp90 (Sullivan et al. 1997). Hop has been shown to bridge and enable the transfer of client proteins from the Hsp70 to the Hsp90 (Smith et al. 1993; Bose et al. 1996; Chang et al. 1997; Zhong et al. 2006). More recently, it has been suggested that Hop is also involved in modulating the activities of Hsp70 and Hsp90 (Gross and Hessefort 1996; Owens-Grillo et al. 1996; Chang et al. 1997; Richter et al. 2003; Wegele et al. 2003) and may bind proteins other than the Hsp70 and Hsp90 chaperones (Glover and Lindquist 1998; Abbas-Terki et al. 2001, 2002). An overview of the Hsp70, Hsp90, and Hop domains and interactions is shown in figure 1.

Hop contains 9 tetratricopeptide repeat (TPR) motifs that are evenly clustered into 3 TPR domains, TPR1, TRP2A, and TPR2B. Both Hsp70 and Hsp90 contain an EEVD motif at their C-termini that are known to interact with the Hop TPR1 and TPR2A motifs, respectively (Scheufler et al. 2000). Recent work, however, suggests that, within Hsp70 and Hsp90, the binding of Hop may not be contained solely within the EEVD motifs (Flom et al. 2005; Carrigan et al. 2006). Odunuga et al. (2004) proposed that the TPR2B domain of Hop may have Hsp70-binding capacity, whereas Song and Masison (2005) suggested that TPR2B may be a Hsp90 ligand. Also, the DP2 repeat region in Hop has been suggested to be important for binding Hsp70 (Karunakaran et al. 2003) and Hsp90 (Flom et al. 2005). These results show that our knowledge of Hop interactions with Hsp70/Hsp90 is far from complete and indicates that a more exhaustive search for binding regions for these chaperones within Hop is required.

Using CAPS, we have investigated the presence of coevolution in amino acid residues between the chaperones Hsp70 and Hsp90 and their cochaperone Hop. We show that previously reported or suggested interacting regions are identified using our method. Further, we have also observed a novel region within Hop coevolving with both Hsp70 and Hsp90. This region has never before been described as important in the function of Hop with Hsp70 and Hsp90, thereby providing an ideal target for future mutagenesis analyses. These results provide support to our method as a useful computational tool in the future identification of protein–protein functional domains.

Materials and Methods

Sequences and Sequence Alignments

Twenty-five eukaryotic organisms for the Hsp70/Hop analysis and 27 organisms for Hsp90/Hop were explored for interprotein coevolution, including unicellular as well as multicellular eukaryotes (table 1, Supplementary

Table 1
Groups of Amino Acid Residues Identified as Coevolving Between Hsp70 and Hop

	Hop Residues													
Hsp70 Residues	TPR1						TPR2B	DP2 Repeat Region					Mean ρ	SE
79P							457I				576I	585I	0.43	0.0526
106K			77Y										0.42	—
113T							457I				576I	585I	0.47	0.0528
136K							457I				576I		0.36	0.0056
191G									525E				0.36	—
236N							457I				576I		0.38	0.0190
246N							457I						0.35	—
275A							457I						0.36	—
315E				228D									0.35	—
319R	2S	4T					457I			567M	576I	585I	0.39	0.0547
394A					229S								0.52	—
412P									525E				0.49	—
542I				228D					525E				0.38	0.0333
543A					229S								0.49	—
546L							457I						0.35	—
552E							457I						0.35	—
565V					229S								0.49	—
569A							457I				576I		0.36	0.0036
579N							457I				576I	585I	0.64	0.1255
583S							457I				576I	585I	0.41	0.0512
589D							457I				576I		0.36	0.0030
597I			77Y										0.38	—
598A							457I				576I		0.35	0.0010
602M								524N					0.35	—
612P									525E				0.37	—
617G				228D		230T			525E				0.48	0.0392
618G											576I	585I	0.36	0.0085
623F								524N	525E				0.49	0.0037
631P								524N	525E				0.47	0.0858
633A							457I				576I		0.42	0.0115

Material online). Individual multiple sequence alignments for Hsp70, Hsp90, and Hop were produced using ClustalW (Bowers et al. 2004), and all alignments were manually edited using MacClade (Maddison WP and Maddison DR 1992) (alignments available from the authors on request). Due to the multiorganism nature of this analysis and in order to avoid confusion, unless otherwise stated, when presenting our results the amino acid numbering corresponds to those of the *Saccharomyces cerevisiae* Hsp70, Sti1, and Hsp90 protein sequences as detailed in table 1 of the Supplementary Material online. The mean amino acid substitution rate per branch and site for each of the proteins is 0.043, 0.034, and 0.093 for the Hsp70, Hsp90, and Hop proteins, respectively. The rate of substitution in the multiple sequence alignments of the proteins used in this study and the number of sequences gave us a predicted sensitivity value following the study of Fares and Travers (2006) of about 97%.

Detecting Intermolecular Coevolution

We examined protein–protein functional coevolution between Hsp and Hop using a parametric method to detect coevolution (Fares and Travers 2006), implemented in the program CAPS version 1.0 (Fares and McNally 2006). Using simulations we have shown that our method greatly improves on the sensitivity of previously published methods to detect intraprotein coevolution and that, even with a large degree of divergence between sequences studied, the method identifies almost 100% of truly coevolving residues and, perhaps more importantly, identifies negligible amounts of false positives (Fares and Travers 2006). This method has been modified and further developed in this study to detect protein–protein evolutionary dependencies in addition to intraprotein amino acid sites' dependencies. We would like to, however, underline the fact that the main purpose of the method is not to detect protein–protein interaction surfaces but rather to highlight functional dependencies between biochemical and physically interacting proteins. The mathematical procedure to detect intramolecular coevolution was previously detailed (Fares and Travers 2006). Because the detection of coevolution between amino acid sites is already normalized by the evolutionary rate of those particular sites (Fares and Travers 2006), this method is also valid for the detection of protein–protein coevolution despite differences in the evolutionary rates of the proteins considered.

Briefly, the method compares the correlated variance of the evolutionary rates at 2 amino acid sites, belonging to the same or different proteins, corrected by the time since the divergence of the species from which protein sequences were obtained. Because of the difference in the amino acid composition and evolutionary rates of the different proteins compared, substitutions or conservation at 2 independent sites cannot be directly compared. The method instead compares the transition probability scores between 2 sequences at these particular sites, using BLOSUM (Blocks Substitution Matrix) (Henikoff S and Henikoff JG 1992). For each

protein alignment the corresponding BLOSUM is applied depending on the average sequence identity. BLOSUM values are then normalized by the divergence time between sequences. For example, BLOSUM values for the transition between 2 amino acids *e* and *k* are weighted using the time (*t*) of divergence between the sequences compared. Finally, the correlation in the amino acid variability between the sites under analysis is measured using the Pearson's correlation coefficient.

To determine if the correlation coefficient (ρ_{AB}) is significant, a simulation analysis can be performed by the Monte Carlo generation of *K* number of simulated sequence alignments that share the same mean parameter values of amino acid proportions, amino acid substitution rates, and phylogenetic relationships between sequences. Simulations were performed using the program evolver from the PAML package version 3.15 (Yang 1997). We then screened simulated sequence alignments for coevolution and calculated the correlation coefficient for pairs of amino acid sites randomly sampled from the simulated multiple sequence alignments. Finally, we tested these correlation coefficients for significance by comparing the real coefficients with the distribution of correlation coefficients generated from the simulated multiple sequence alignments.

To account for multiple tests and nonindependence of the data, we applied the step-down permutational procedure (Westfall and Young 1993). In order to improve the statistical confidence of our results, we dropped the confidence level (type I error) to 0.001 instead of the 0.05 normally used. We then sorted these significant correlation coefficients and included the coefficients obtained from the real sequence alignment in the sorted list. The probability of the real coefficients was finally calculated by dividing their position in the sorted list by the total number of correlation coefficients (the protocol for the filtering of the correlation coefficients is shown in fig. 2).

Identifying Functionally Important Protein Domains

Coevolution between 3-dimensionally distant sites is usually observed when these sites are surrounding functionally important regions. In these cases, coevolution between amino acid sites mainly occurs to maintain the structural characteristics around these regions and consequently to maintain the conformational and functional stability of the domain (Gloor et al. 2005). Even though circular, the opposite reasoning can be used for a primary detection of functionally/structurally important protein sites or domains. We therefore used this rationale to identify functionally important sites nearby coevolving residues. For example, when a residue was involved in interprotein coevolution, we examined whether functionally important sites nearby any of the coevolving amino acid regions (less than 8 Å distance from the functionally important region) was previously reported. If that was the case, we supported the usefulness of the method to detect functional domains and proposed new domains for mutagenesis experiments. This approach is also valid, hence, for identifying functionally important sites that show very low evolutionary rate and that would therefore be completely ignored by standard coevolutionary analyses.

Results

In our previous study (Fares and Travers 2006), we developed a method to detect intraprotein evolutionary dependencies between amino acid sites. Here we have extended this method to detect protein–protein coevolutionary networks (details about the method algorithm are presented in Materials and Methods and fig. 2). Analyses of simulated sequence alignments with interprotein coevolutionary relationships show that the method presents similar sensitivity values compared with intramolecular analyses (sensitivity values ranging between 70% and 90% in multiple sequence alignments comprising 10 sequences and between 86% and 97% in multiple sequence alignments containing 20 or more sequences).

Coevolution between Hop and Hsp70

Application of interprotein CAPS analysis using 25 eukaryote Hsp70 and Hop sequences identified the presence of a number of groups of coevolving amino acid residues (table 1). Within Hsp70, 30 amino acid residues were identified as coevolving with amino acid residues in Hop, with the correlation coefficients ranging between a minimum value of 0.35 and a maximum value of 0.73 (both correlation coefficients with probabilities of $P < 0.001$; table 1). The majority of these coevolving residues were located in either the NBD or the C-terminal domain with only 5 residues (394A, 412P, 542I, 543A and 546L) identified in the SBD. In total, 50% of all the residues within Hsp70 identified as coevolving with Hop were located in the C-terminal domain, the domain known to be involved in functional interactions with Hop. Interestingly, amino acid residues from different domains within Hsp70 were observed as coevolving with the same residues in Hop, suggesting that there is a high codependence of evolution between the functional domains of Hsp70.

Of the 30 amino acids in Hsp70 identified as coevolving with residues in Hop, one-third of these are proximal in the 3-dimensional structure to residues in where mutations somehow affect Hsp70 function. Coevolving residues are observed as clustering together in groups within the bovine Hsc70 3-dimensional structure (Jiang et al. 2005) (fig. 3). Although not proximal to each other in the 3-dimensional structure, amino acid residues 79P and 236N, both coevolving with residues in the TPR2B and DP2 regions of Hop (table 1), are directly proximal to residues that comprise part of a multiple bacterial Hsp70 homologue (DnaK) mutant able to restore growth at high temperatures of cells containing a D35N mutant in bacterial Hsp40 homologue (DnaJ) (Suh et al. 1998). Residues 315E and 319R, while coevolving with different residues in Hop (table 1), are both directly adjacent to residues 322K and 527E, the mutation of which affects the ability of Hsc70 to bind auxilin, to disassemble clathrin cages and to hydrolyze ATP (Jiang et al. 2005) (fig. 3). Both residues, 322K and 527E, seem to be functionally/structurally linked because the effects of mutating one residue can be compensated by the mutation of the second (Jiang et al. 2005). Within the SBD, residues 394A and 412P (table 1) are both significantly proximal to residues 395P and 413R, which, in turn, are adjacent to 213I, 512I, and 516V (fig. 3). The mutant P395L in yeast

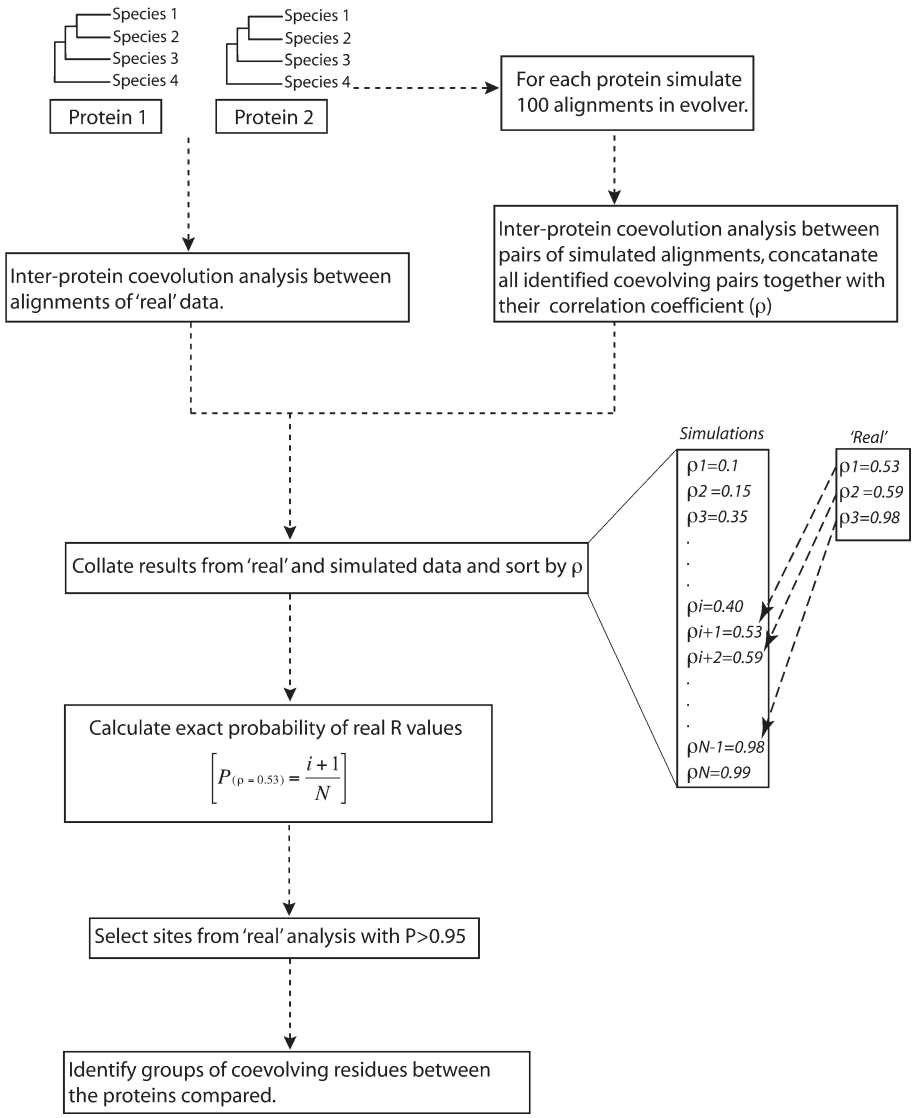

FIG. 2.—Flow chart detailing the identification of significantly coevolving residues in "real" data using simulations.

abolishes (URE3) prion propagation (Roberts et al. 2004), whereas the 413R mutant in DnaK (K414I in DnaK numbering) cannot support phage λ replication in vivo and also shows an absence of allosteric function in vitro (Pellecchia et al. 2000). Jiang et al. (2005) identified 213I, 512I, and 516I as important in interdomain communication within Hsc70. Finally, 542I, 543A, and 546L (fig. 3) are proximal to hydrophobic residues (403A, 427T, 428Y, 440F, and 447T) identified as important in the binding of substrates by Hsp70 (Pellecchia et al. 2000).

The TPR1 and C-terminal DP2 repeat regions in Hop are known to be important for Hop–Hsp70 binding (Scheufler et al. 2000; Karunakaran et al. 2003), and amino acids in both these regions were observed as coevolving with Hsp70 (TPR1: 4T and 77Y; DP2: 524N, 525E, 563L, 576I, and 585I). Also, site 457I in the Hop TPR2B domain was observed as coevolving with sites in the NBD and C-terminal domain of Hsp70 (table 1). Apart from the 2 TPR domains and the C-terminal DP2 repeat domains, only one other region of site in Hop was identified as coevolving with Hsp70 (table 1). These residues (228D, 229S, and 230T) are located in a region of approximately 56 amino acid residues (in yeast Sti1) following the DP1 region between the TPR1 and TPR2A domains in Hop (hereafter described as the TPR linker region), a region not previously reported as being involved in Hsp70–Hop interaction.

The only available structural data for Hop are those of the TPR1 domain bound with the Hsc70 EEVD peptide (fig. 4) and the TPR2 domain bound with the Hsp90 MEEVD peptide (Scheufler et al. 2000). In the absence of complete 3-dimensional data, identifying the proximity of all amino acid residues to each other is impossible. However, of the residues in Hop identified as coevolving with Hsp70, position 77Y in TPR1, coevolving with 106K and 597I in Hsp70, is directly adjacent to 75K and 76G (fig. 4). These residues were proposed by Song and Masison (2005) as being critical for Sti1 (Hop in yeast) regulation of Hsp70, whereas other studies have confirmed 75K as a site critical for Hsp70 binding (Flom et al. 2005; Carrigan et al. 2006) along with 79R (Flom et al. 2005).

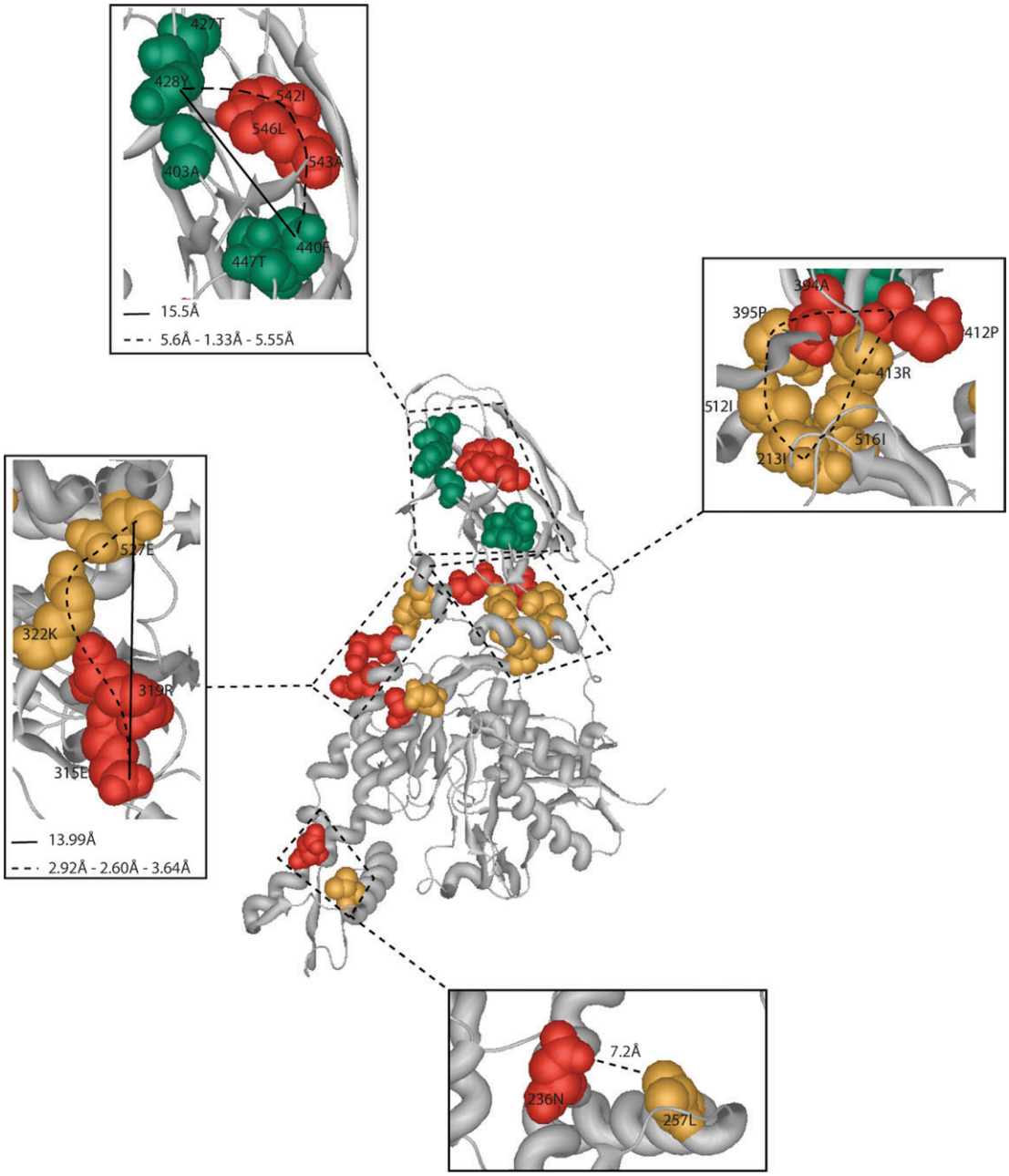

FIG. 3.—Amino acid residues in the bovine Hsp70 structure identified as coevolving with residues in Hop (red). Also shown are residues at which mutation has been shown to affect Hsp70 function (yellow) and hydrophobic residues that have been implicated as important in the binding of substrates by Hsp70 (green). The proximity within groups of amino acid residues is shown in the expansion panels showing the distance in Angstroms (Å). Dashed lines indicate the coevolutionary pathway connecting 2 amino acids. For example, amino acid residues 428Y and 440F, both involved in the binding of substrates by Hsp70, are linearly distant in the 3-dimensional structure (solid line). Their distance becomes much smaller when the amino acids coevolving in the proximity of that region are taken into account. In this case, 440F is very close to the coevolving amino acids 542I, 546L, and 543A, which in turn are close to 428Y (dashed line).

Coevolution between Hop and Hsp90

We performed the interprotein coevolution analysis between Hsp90 and Hop using 27 eukaryote sequences for each protein. Within Hsp90, 51 amino acid residues, representing all the major domains within Hsp90, were observed as coevolving with Hop and presented correlation coefficients ranging between a minimum value of 0.33 and a maximum value of 0.75 ($P < 0.001$; table 2). The majority of residues in Hsp90 coevolving with Hop are located within the NBD and the middle segment (table 2). Interdomain communication is known to be essential for the operation of Hsp90 (Meyer et al. 2003), and the observation of a large degree of overlap of shared coevolving sites with Hop between the various domains of Hsp90 reflects this (table 2). Three-dimensional structures have been resolved for the Hsp90 NBD (Prodromou, Roe, O'Brien et al. 1997; Stebbins et al. 1997) and middle domain (Meyer

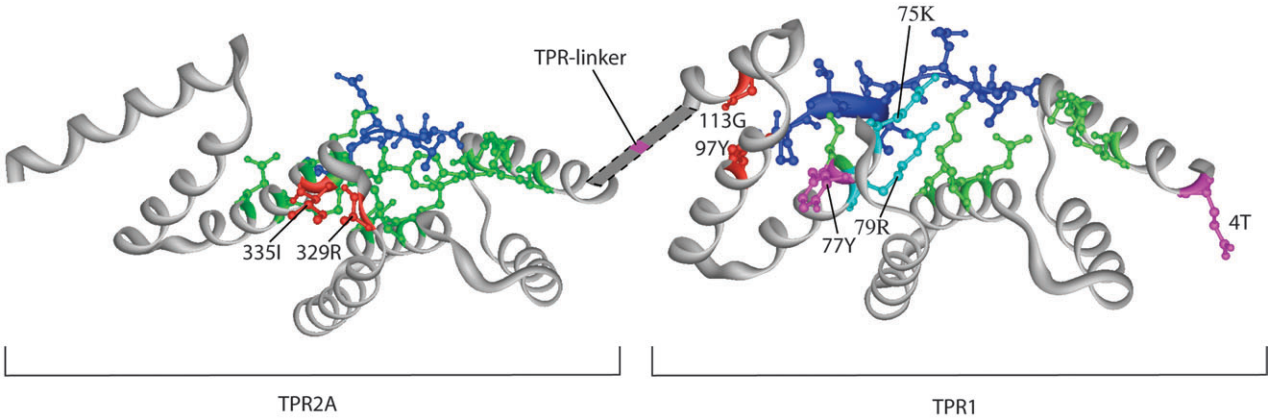

FIG. 4.—The TPR1 and TPR2A domains of Hop bound with their respective Hsc70 EEVD and Hsp90 MEEVD peptides (dark blue) (Scheufler et al. 2000). TPR residues known to bind directly to the peptides are marked (green) as are residues at which mutation affects Hop–Hsp70 interaction (Flom et al. 2005; Song and Masison 2005; Carrigan et al. 2006) (turquoise). Residues identified as coevolving with Hsp90 are marked (red) along with residues identified as coevolving with both Hsp70 and Hsp90 (magenta). It is to be noted that positions 75K and 79R also interact directly with the Hsp70 EEVD peptide. A cartoon of the TPR linker domain, in which residues were identified as coevolving with Hsp90 (table 2), is also shown for clarity.

et al. 2003). Of the 51 amino acid residues in Hsp90 coevolving with Hop, 15 are within the NBD structure, whereas 13 are within the middle segment structure (fig. 5).

When mapped on the Hsp90 NBD structure (Prodromou, Roe, O'Brien et al. 1997), 14 of the 15 residues identified as coevolving with Hop are localized to the 8-stranded β sheet adjacent to the ATP-binding pocket (Prodromou, Roe, Piper, Pearl 1997) (fig. 5). The one exception is 110A, which is located within the flexible lid proposed to close over, and interact with, bound ATP (Prodromou, Roe, O'Brien et al. 1997). The Hsp90 middle domain exhibits significant divergence in 3-dimensional structure from the equivalent regions of other related proteins, such as MutL DNA mismatch repair protein and DNA gyrase B (GyrB), in that it contains an additional domain (Meyer et al. 2003). Interestingly, the majority of residues within the Hsp90 middle segment coevolving with Hop are clustered within this novel domain (fig. 5). Residues within the Hsp90 novel domain are observed as coevolving with residues in the TPR1, TPR2A, TPR2B, DP2, and TPR linker domains of Hop (table 2). Within this novel Hsp90 region, residues 519D and 524F, both coevolving with residues 228D and 230T (TPR linker) and 525E (DP2) in Hop, are directly adjacent to 525T (fig. 5), the mutation of which detrimentally affects Hsp90 interaction with glucocorticoid (GR), mineralocorticoid, estrogen, and progesterone receptors (Bohen and Yamamoto 1993). Also in the Hsp90 middle region, residue 429T, coevolving with residues 228D and 229S in the Hop TPR linker domain, is directly adjacent to 431E (fig. 5), previously identified as necessary for GR binding (Bohen and Yamamoto 1993).

Amino acid residues from all the TPR domains within Hop are observed as coevolving with Hsp90, as are residues in the TPR linker region and the C-terminal DP2 repeat region (table 2). The Hop TPR1 residues (4T, 77Y, 97Y, and 113G) and TPR2A residues (329R and 335I), identified as coevolving with Hsp90, were plotted on their respective three-dimensional structures (fig. 4). Two of the 4 residues within the TPR1 domain identified as coevolving with Hsp90 were also observed coevolving with Hsp70 (fig. 4). The other 2 residues within TPR1, 97Y, and 113G are proximal to each other as well as to 77Y (fig. 4). The crystal structures of TPR1 and TPR2A are highly similar, with the bound peptides in both TPR1 and TPR2A making contact in similar positions with side chains of the same helices in the structure (Scheufler et al. 2000) (fig. 4). The residues within Hop TPR2A identified as coevolving with Hsp90 (329R and 335I) are located in the region involved in interaction with the Hsp90 peptide (fig. 4). Interestingly, 335I appears to be in a similar position in TPR2A as 77Y is in TPR1, directly adjacent to residues essential in the interaction between the TPR and its respective Hsp peptide (fig. 4).

Discussion

A Powerful Computational Method to Detect Proteins Functional Coevolutionary Networks

In this study, we present the application of a powerful computational tool to identify functional coevolutionary networks among proteins within a specific cellular pathway. Our analysis of functional dependencies between proteins in an apparently well-characterized cell cycle leads to a number of conclusions. First, the method is proven powerful as known amino acid regions involved in protein–protein interactions within the Hsp70–Hop–Hsp90 system have been identified. Second, regions previously suggested as important for such interactions (whether in direct interactions or otherwise) have been pinpointed by the method, and, finally, we have identified novel regions that we suggest are involved in the functional relationships between the proteins studied. We propose these regions as a priori targets for mutagenesis experiments and we predict their functional importance in the stability of the Hsp70–Hop–Hsp90 cycle. Other studies have conducted similar approaches but with different focus. For instance, coevolutionary analysis has been used to predict protein folds through the study of interatomic evolutionary interactions (Socolich et al. 2005). This approach was also used on protein design (Poole and Ranganathan 2006). The main differences between their

Table 2
Groups of Amino Acid Residues Identified as Coevolving Between Hsp90 and Hop

	Hop Residues																		
Hsp90 Residues			TPR1							TPR2A		TPR2B		DP2 Repeat Region				Mean ρ	SE
7E						228D		230T							525E			0.39	0.0554
9Q							229S											0.33	—
13T														524N				0.42	—
54K														524N				0.42	—
60P						228D	229S	230T					523S	524N	525E			0.44	0.1164
67T						228D												0.42	—
70P						228D								524N				0.37	0.0232
73K							229S											0.34	—
78R							229S											0.36	—
110A											335I							0.35	—
133R						228D	229S											0.38	0.0085
151N						228D		230T							525E			0.47	0.0439
163V							229S							524N				0.52	0.1493
174R						228D		230T							525E			0.41	0.0741
209V							229S											0.59	—
215K										329R								0.34	—
218P						228D	229S	230T	231T					524N	525E			0.45	0.1523
223E										329R								0.33	—
232E						228D												0.36	—
234D						228D	229S	230T							525E			0.40	0.1099
239L			97Y	113G							335I							0.44	0.1076
244E						228D												0.35	—
246E						228D												0.45	—
257K						228D	229S											0.34	0.0034
261Q							229S											0.39	—
263L							229S							524N				0.40	0.0019
314R											335I							0.33	—
401N								230T							525E			0.45	0.1071
429T						228D	229S											0.45	0.0266
444T						228D												0.33	—
463H								230T							525E			0.49	0.1155
482P						228D		230T	231T				523S	524N	525E			0.45	0.1533
488K											335I							0.34	—
492F								230T							525E			0.37	0.0610
505A	4T				147K													0.49	0.2040
506F														524N				0.42	—
514G						228D												0.34	—
519D						228D		230T							525E			0.42	0.0846
524F						228D		230T							525E			0.50	0.0940
526L												457I				576I		0.37	0.0080
543Y											335I							0.37	—
566K												457I				576I		0.35	0.0117
568L		77Y																0.34	—
569D								230T							525E			0.39	0.0566
577G						228D		230T	231T						525E			0.51	0.1661
598S						228D												0.35	—
609F						228D		230T						524N	525E			0.48	0.1430
628G						228D												0.36	—
634T						228D												0.37	—
639T						228D												0.36	—
662S	4T															576I	585I	0.41	0.1436

approach and our method are that their method is designed for intramolecular analysis, requires highly populated multiple sequence alignments, and is focused on predicting protein local folds and in protein design.

Despite the fact that our method is not focused on the detection of physical dependencies but rather functional dependencies, studies of coevolution between interacting and noninteracting proteins show a clear positive correlation between the strength of coevolution (correlation coefficient) between amino acids and their physical interaction when comparing both sets of data (data not shown). In addition, proteins that do not interact from the functional point of view (do not belong to the same pathway) and physical point of view show no parsimony-informative coevolution signal when both the stochastic and phylogenetic coevolutions are removed from results (data not shown).

Coevolution Identified between Hsp70 and Hop Shows Not Only Expected But Also Novel Results

Although previous studies have suggested a number of regions within Hop which are important for its interaction with Hsp70 (Scheufler et al. 2000; Karunakaran et al. 2003; Odunuga et al. 2004; Song and Masison 2005), our

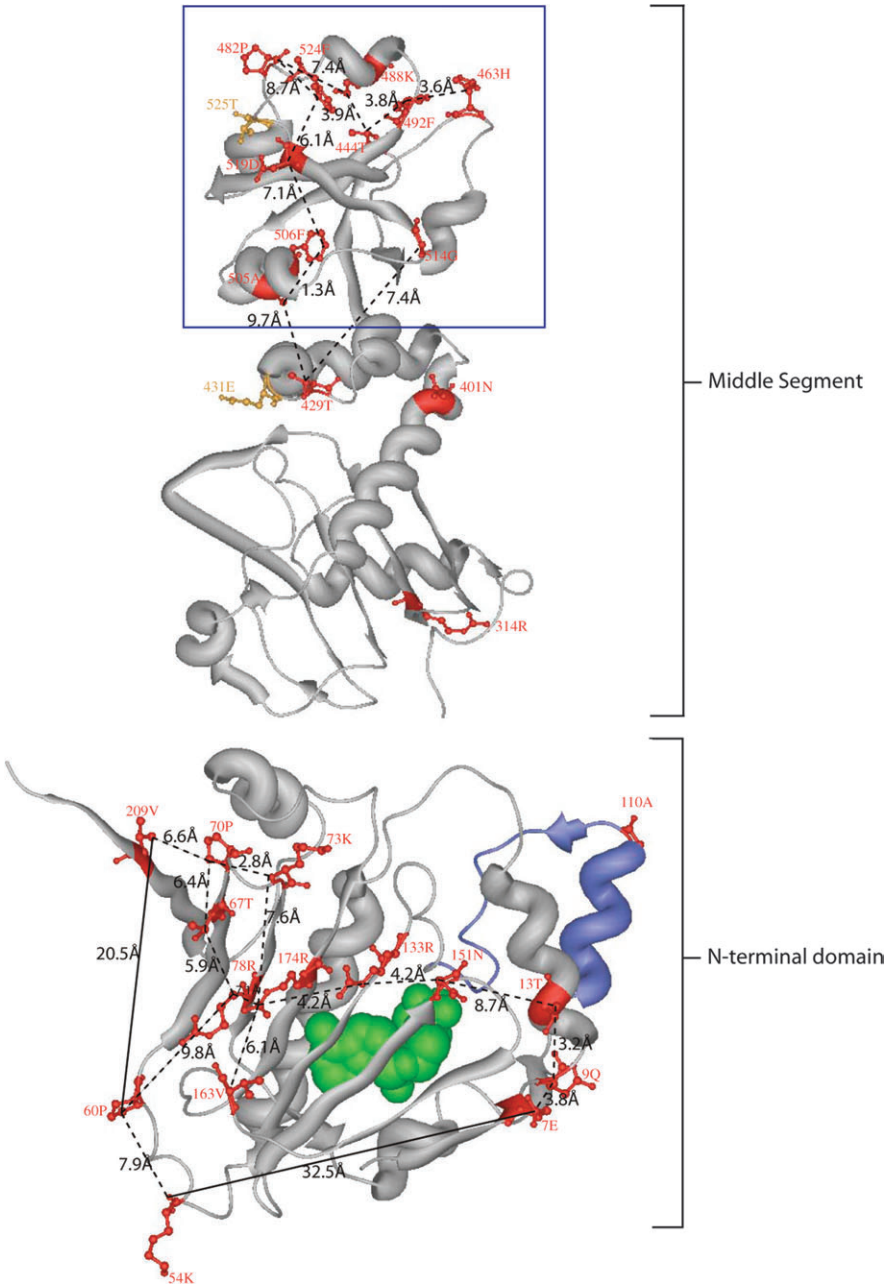

FIG. 5.—The Hsp90 N-terminal (Prodromou, Roe, Piper, Pearl 1997) and middle segment (Meyer et al. 2003) structures. Residues identified as coevolving with Hop are shown in both structures (red), as are bound ADP (green) and the proposed lid segment (blue) within the N-terminal structure. Residues previously identified as important in substrate binding (Bohen and Yamamoto 1993) that are structurally proximal to residues identified in this analysis as coevolving with Hop are also shown in the middle segment (orange). In both structures the proximity of residues identified as coevolving with Hop is shown. The domain unique to Hsp90 (Meyer et al. 2003) is shown within a blue frame.

understanding of the interactions between these 2 proteins is far from complete. Our analysis has identified groups of amino acid positions within Hsp70 and Hop that are evolving in a codependent fashion. Although coevolution is not an indication of protein–protein interaction at these positions, the identification of coevolution at these residues is indicative of some manner of dependence (functional, structural, or energetic interaction) in the evolution of these proteins. Coevolution between amino acid sites can be also indirect, for example, due to their functional/structural link to the same active site (Gloor et al. 2005).

The observation of coevolution between regions in Hop known to be important for the interaction with Hsp70 provides support for the sensitivity of our method in detecting domains involved in protein–protein interaction. We identified coevolution between the TPR1 domain and the C-terminal DP2 repeat domain in Hop, both known to be important for the interaction of Hop and Hsp70 (Scheufler et al. 2000; Karunakaran et al. 2003). Methods to detect coevolution rely on the amount of variability information to infer covariation between 2 amino acid sites. Conserved residues (e.g., amino acid sites with no amino

acid mutations) would not be identified as coevolving despite their possible interaction. Unsurprisingly, due to its conservation across all species, we did not observe coevolution between the EEVD motif in the Hsp70 C-terminal and the TPR1 domain of Hop, which are known to interact (Scheufler et al. 2000).

Our results are highly conservative in that they identify regions within Hop known to be important for Hsp70 binding (TPR1 and the C-terminal DP repeat region) and regions hypothesized to be necessary for Hsp70 binding (TPR2B). The only other residues identified in Hop as coevolving with Hsp70 are located between the TPR1 and TPR2A domains (228D, 229S, and 230T).

Coevolution between Hsp90 and Hop Underlines the Need for Further Protein–Protein Interaction Analyses

Currently, it is known that Hsp90 interacts with the TPR2A domain of Hop and it has also been suggested that Hop TPR2B may act as an Hsp90 ligand and that DP2 may be involved in Hsp90 interaction (Flom et al. 2005; Song and Masison 2005). Therefore, the identification of coevolution between amino acids within these domains and residues in Hsp90 is not unexpected (table 2). With the exception of 110A, all the residues within the Hsp90 ATPase domain identified as coevolving with Hop are localized to the 8-stranded β sheet proximal to the ATP-binding pocket (Prodromou, Roe, Piper, Pearl 1997). Of these residues, 78R and 174R are directly adjacent to residues comprising the ATP-binding pocket, 2 of which (79D and 171T) are observed as directly interacting with bound ADP (Prodromou, Roe, O'Brien et al. 1997). In fact mutation at 79D has been shown to affect ATP binding and hydrolysis in Hsp90, resulting in a failure to maintain cell viability in vitro (Ali et al. 2006). These results are consistent with the observation by Gloor et al. (2005) that coevolving sites are frequently located in regions critical for protein function such as active sites. It is well documented that the ATPase activity of Hsp90 is regulated by Hop (Prodromou et al. 1999) and, perhaps, the observed coevolution between the Hsp90 N-domain and Hop occurred in order to maintain 3-dimensional structures in an optimal conformation for the regulation of ATP hydrolysis. Interestingly, the only other residue within the NBD of Hsp90 identified as coevolving with Hop, is located on the lid segment of the NBD which is hypothesized to close following ATP binding which, in turn, promotes N-terminal dimerization, thereby resulting in the closure of the clamp required for optimal ATPase activity (Prodromou et al. 2000).

Surprisingly, the majority of residues within the middle segment of Hsp90 observed as coevolving with Hop are located in the Hsp90-unique domain identified in comparison with MutL and GyrB structures (Meyer et al. 2003) (fig. 5). Within this domain, mutation at residue T525, in yeast Hsp90, affected Hsp90 interaction with a number of client proteins (Bohen and Yamamoto 1993), whereas mutation at the corresponding position in mouse Hsp90 (T541) affected the interaction of Hsp90 with the FKBP51 and p23 cochaperones (Zhong et al. 2006). We observe a large degree of coevolution between this domain and Hop, and we think that the relationship between this novel region and Hop should be studied further.

Shared Coevolution between Hsp70, Hsp90, and Hop

In a system such as the Hsp70–Hop–Hsp90 system, in which the optimal function of the system depends on complex intramolecular and protein–protein interactions and chains of interactions between its constituent proteins, changes within the domain of one protein can have a knock-on affect on the domain of another protein, even if the 2 domains are not directly interacting. This implies that compensatory changes are needed elsewhere within the system in order to maintain its stability. Therefore, it is not surprising that we observe coevolution between domains of proteins not previously reported to be interacting with, or regulating, each other. In the same vein, the observation of a large degree of overlap in residues within Hop coevolving with residues in both Hsp70 and Hsp90 is not surprising. For example, the TPR1 domain of Hop is proposed to be important for the binding of Hsp70, yet we identified 4 residues within TPR1 as coevolving with Hsp90, 2 of which overlap with those identified as coevolving with Hsp70 (tables 1 and 2). On the other hand, amino acid sites from Hsp90 and Hsp70 detected to be coevolving with the same sites in Hop are also detected to be coevolving between each other when we perform analysis of coevolution between Hsp90 and Hsp70. Also, sites coevolving in intraprotein coevolutionary analyses in Hsp70 or Hsp90 can be also detected in interprotein analyses of these proteins with Hop despite their indirect involvement in the physical interaction between the proteins. Although this problem is unavoidable given the limitation to distinguish between physical and functional coevolution, this approach can also be used to detect functionally related amino acid sites within a protein or between proteins. In such a complex system, as with most protein systems, it is incredibly difficult to categorize coevolving residues (interacting, structural, functional) and to distinguish between direct and indirect interaction. The reason for such difficulty is that if protein A is interacting with protein B and B with C, we would expect certain transitivity in the system such as A coevolves with C. This means that the indirect coevolution between proteins may provide overlapping coevolving amino acid residues between any protein pairs of the system. Future work in this field should aim to extrapolate the various classes of coevolving residues within a protein system. If one can, in fact, separate the interacting, structural and functional classes of coevolving residues within a protein system, it is therefore theoretically possible to utilize coevolution analyses in the prediction of protein–protein interaction. Also, identifying key coevolving amino acid residues between Hsp and their known clients may provide a mechanism for the computational identification of further, as yet unknown, Hsp clients.

Proximity of Coevolving Residues to Functionally Important Residues

Most proteins exhibit residues at positions within the amino acid sequence that have been conserved throughout the evolutionary history due to functional constraints.

Natural selection is responsible for this conservation, as a change at these residues will have a negative affect on protein function. The identification of such functionally conserved residues using coevolution methods is impossible, as no variability can be measured at these sites and hence no information exists to conduct powerful statistical tests. All functional sites, however, are not conserved throughout evolutionary history and, therefore, the in silico prediction of functional residues within proteins remains a realistic endeavor. In these analyses, we have observed many coevolving sites to be spatially proximal to residues previously implicated to be of functional importance. Therefore, it is plausible to suggest that mutation of highly conserved amino acids 3-dimensionally proximal to residues identified as coevolving may, in fact, enable the identification of highly conserved functionally important sites within proteins.

The Identification of a Novel Putative Functional Domain within Hop?

With one exception, all the domains within Hop identified as coevolving with Hsp70 and/or Hsp90 have been previously identified as functionally important in interactions within the Hsp70–Hop–Hsp90 protein–folding system. In our analysis we have observed one region within Hop, termed here as the TPR linker domain, which has not been previously shown to be important for the interaction of Hop with Hsp70/Hsp90. The TPR linker domain is not conserved within lower eukaryotes, whereas it is extremely conserved between higher eukaryotes. In fact, within this domain, when comparing Hop sequences from higher and lower eukaryotes, using the computer software DIVERGE (Gu and Vander Velden 2002), we identified 2 residues (202N and 206S) as having undergone functional divergence type I (altered evolutionary rates at amino acids in higher eukaryotes when compared with lower eukaryotes, due to novel functional constraints within higher eukaryotes). Although no 3-dimensional structures are available for Hop, neural network analysis using the computer program CONSEQ (Berezin et al. 2004) predicted that the TPR linker is exposed on the molecule and also that a number of residues within this domain are functionally important in both *S. cerevisiae* and *Homo sapiens* Sti1/Hop (data not shown). In the absence of conclusive mutagenesis data and 3-dimensional structures, it is difficult to definitively propose a function for this TPR linker domain, but we feel that, due to its proximity to the TPR1 and TPR2A domains in Hop, it may play a role in binding and/or regulation of Hsp70 and Hsp90 interactions with Hop. Our proposal of a function for the TPR linker region was purely based on the in silico analysis performed in this study.

However, following the in silico analysis, we uncovered data by Longshaw et al. (2004) proposing that this region may, in fact, perform a role in Hop binding to Hsp70/Hsp90. Within our proposed TPR linker domain, they identified a casein kinase II (CKII) phosphorylation site and a cdc2 kinase phosphorylation site at positions 189S and 198T, respectively, in murine Hop. They observed that mimicking phosphorylation at 189S promotes nuclear localization of Hop, whereas phosphorylation at 198T promotes cytoplasmic localization of Hop at the G1/S-phase transition. Although the mutations at these positions did not seem to affect Hop binding with Hsp70 and Hsp90, they suggested that phosphorylation at these positions may be involved in regulation of the nuclear localization signal and therefore indirectly in the assembly of Hsp70–Hop–Hsp90 complexes.

Taking into account the conclusions of Longshaw et al. (2004) and the observation, in this study, of coevolution only within functionally important domains in Hop, we propose that this TPR linker domain is, in fact, functionally important within the Hsp70–Hop–Hsp90 complexes. The fact that many coevolving sites are neighboring or directly contacting functionally important regions or amino acid sites previously proposed is due to that coevolutionary analyses are based upon the presence of evolutionary information. This type of analyses therefore misses highly conserved sites. The a priori assumption of our method that considers important those coevolving sites surrounding functionally important and highly conserved regions (see Materials and Methods) ameliorates this problem and permits the detection of functional sites in the protein. We think that further mutagenesis analysis should be performed in definitively defining the role of this region within the Hsp70–Hop–Hsp90 protein–folding system. We suggest that these analyses should be performed in organisms from both higher and lower eukaryotes as we have observed a degree of functional divergence within this TPR linker domain between them.

Supplementary Material

Supplementary materials are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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