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[26] Identifying Functional Interactions with Molecular Chaperones

By JILL L. JOHNSON and ELIZABETH A. CRAIG

All organisms have proteins that function in the maturation of other proteins, including their folding and translocation across membranes. *Saccharomyces cerevisiae* is no exception, as it has molecular chaperones in all major cellular compartments. Some are induced by stresses such as increased temperatures, while expression and function of others are constitutive. Many investigators studying diverse cellular processes encounter molecular chaperones in the course of their work. The challenge facing these scientists is whether the interactions observed are biologically meaningful or if they are artifacts caused by the propensity of molecular chaperones to interact with hydrophobic stretches of proteins, particularly of partially unfolded proteins. This is not an easy question for anyone to answer. However, this chapter describes tools and approaches that can be used to address the relevance of such interactions.

Heat Shock (Stress)-Inducible Genes

The highly conserved molecular chaperones called heat shock proteins (Hsps) are divided into classes. As this classification was originally done according to their migration in SDS-PAGE gels, the major groupings are called (somewhat inaccurately), Hsp104, Hsp90, Hsp70, Hsp60, and "small Hsps" (Hsp26). Although some of these proteins are encoded by single genes, others form very complex multigene families. For example, the cytosolic chaperones Hsp104 and Hsp26 are encoded by single genes. However, a mitochondrial protein related to Hsp104, called Hsp78, also exists in yeast. Hsp90 is a collective name for two closely related cytosolic proteins: the heat-inducible Hsp82 and the constitutively expressed Hsc82. The name Hsp70 encompasses 14 different proteins, including two members of the Hsp110 subfamily, Sse1/2. As an additional layer of complexity, many of these chaperones work with cochaperones, e.g., Hsp70s function with Hsp40s (DnaJ proteins). In addition, a number of heat-inducible genes of *S. cerevisiae* are not obviously related to the ubiquitous heat shock proteins that have been highly conserved, some of which may be molecular chaperones as well.

More detailed information about the individual yeast chaperone families can be found in the volume "Guidebook to Molecular Chaperones and Protein-Folding Catalysts," edited by Gething,¹ as well as reviews that have references to many

¹ M.-J. Gething, "Guidebook to Molecular Chaperones and Protein-Folding Catalysts." Oxford Univ. Press, New York, 1997.

relevant papers.²⁻⁷ In addition, the *Saccharomyces* Genome Database⁸ and the Proteome YPD database⁹ are excellent references for finding information about gene expression and known protein–protein interactions of all yeast proteins. However, this chapter focuses on Hsp70s and Hsp90s, the general chaperones most commonly found when studying other cellular processes.

Test for Induction of Heat Shock (Stress) Response

Some investigators actively study the heat shock response and its physiological consequences. Probably many more do not realize that the heat shock/stress response is active under their experimental conditions. For example, conversion of yeast cells to spheroplasts often results in the induction of heat shock genes.¹⁰ Two types of transcription factors function to regulate the response of *S. cerevisiae* to stress: the “heat shock factor,” Hsf1, and “general stress response factors,” Msn2 and Msn4. Hsf1 binds to promoter elements termed HSEs, whereas Msn2/4 bind to STRE elements (reviewed in Estruch¹¹). Induction of stress genes by these transcription factors may occur due to stresses other than an increase in temperature. Induction by Hsf1 is triggered by other factors, such as accumulation of abnormally folded proteins, particularly in the cytosol/nucleus, high concentrations of ethanol, and perhaps oxidative stress. Msn2/4 are activated not only by heat shock and ethanol, but are also under negative control by protein kinase A. Thus, as cyclic AMP levels fall due to nutrient limitation, particularly as cells approach stationary phase, genes containing STREs in their promoters are activated. In addition, such genes are responsive to osmotic stress via the *HOG1* pathway. Several approaches can be used to determine whether particular conditions induce the expression of stress-inducible genes: promoter fusions to indirectly measure expression from a stress-inducible promoter or RNA (Northern) blots or immunoblots to measure the expression of a particular mRNA or protein.

² K. Morano, P. Liu, and D. Thiele, *Curr. Opin. Microbiol.* **1**, 197 (1998).

³ B. Bukau and A. L. Horwich, *Cell* **92**, 351 (1998).

⁴ M. Leroux and F. Hartl, *Curr. Biol.* **10**, 260 (2000).

⁵ E. Craig, W. Yan, and P. James, in “Molecular Chaperones and Folding Catalysts: Regulation, Cellular Function and Mechanisms” (B. Bukau, ed.), p. 139. Harwood Academic, Amsterdam, 1999.

⁶ L. H. Pearl and C. Prodromou, *Curr. Opin. Struct. Biol.* **10**, 46 (2000).

⁷ M. P. Mayer and B. Bukau, *Curr. Biol.* **9**, R322 (1999).

⁸ J. M. Cherry, C. Ball, K. Dolinski, S. Dwight, M. Harris, J. C. Matese, G. Sherlock, G. Binkley, H. Jin, S. Weng, and D. Botstein, <http://genome-www.stanford.edu/Saccharomyces/>.

⁹ M. C. Costanzo, M. E. Crawford, J. E. Hirschman, J. E. Kranz, P. Olsen, L. S. Robertson, M. S. Skrzypek, B. R. Braun, K. L. Hopkins, P. Kondu, C. Lengieza, J. E. Lew-Smith, M. Tillberg, and J. I. Garrels, *Nucleic Acid. Res.* **29**, 75 (2001).

¹⁰ C. Adams and D. Gross, *Mol. Cell. Biol.* **173**, 7429 (1991).

¹¹ F. Estruch, *FEMS Microbiol. Rev.* **24**, 469 (2000).

Promoter Fusions

The use of promoter fusions has the advantage of ease of measurements, as fusions to *lacZ*, encoding the enzyme β -galactosidase, whose activity is measured very easily, are commonly employed.¹² However, each strain must be transformed with the plasmid carrying the gene fusion and selective pressure must be applied to maintain the plasmids containing the constructs, unless they are integrated into the chromosome. The fusion we find particularly useful is a translational fusion between the promoter of *SSA4* encoding a cytosolic Hsp70 and *lacZ* (pWB213, a centromeric plasmid carrying the *TRP1* gene¹³). *SSA4* is the archetypal heat shock gene. It is expressed at extremely low levels under optimal growth conditions and is induced greater than 100-fold under stress conditions. *SSA4* is a sensitive indicator of cell stress, as we have seen subtle stress conditions where *SSA4* shows substantial induction and other heat-inducible genes do not. However, it should be remembered that *Ssa4* has HSEs in its promoter, but not STRE elements, and is thus regulated by Hsf1, but not Msn2/4.

CTT1, encoding catalase T, is regulated by Msn2/4. Analysis of fusions to *lacZ* integrated into the chromosome at the *URA3* locus (pTB3 and derivatives) have been described (e.g., in Wieser *et al.*¹⁴). Fusions of 390 bp of the *CTT1* promoter to *lacZ* show a 35-fold induction after a heat shock from 23 to 37°. It should be kept in mind that the *CTT1* promoter contains promoter elements in addition to STREs, including a binding site for the heme-regulated transcription factor Hap1. However, a segment of the promoter-325 to 382 acts as a UAS in the context of the *LEU2* promoter; this fusion (AW2X) is 15-fold inducible by heat shock.

Two stress-inducible genes, *HSP104* and the Hsp70 gene, *SSA3*, appear to be regulated by both systems, although in the case of *SSA3* this regulation is complex.¹¹ *lacZ* fusions have been used to measure expression from the promoters of both these genes.^{15,16} The *HSP104* : *lacZ* fusion showed a 8- to 10-fold induction on a heat shock of cells grown on glucose-based media. This fusion was constructed in the *URA3*-based integrative *lacZ* fusion vector YIP358R.¹⁷ The *SSA3* promoter fusion, carried on a centromeric vector harboring the *TRP1* gene, has been characterized more thoroughly.¹⁸ A wild-type strain containing a *SSA3* : *lacZ* translational fusion (pWB204 Δ -583) and growing logarithmically on glucose-based media has approximately 3.5 units of β -galactosidase activity. On heat shock the activity

¹² C. M. Nicolet and E. A. Craig, *Methods Enzymol.* **194**, 710 (1991).

¹³ W. R. Boorstein and E. A. Craig, *J. Biol. Chem.* **265**, 18912 (1990).

¹⁴ R. Wieser, G. Adam, A. Wagner, C. Schuller, G. Marchler, H. Ruis, Z. Krawiec, and T. Bilinski, *J. Biol. Chem.* **266**, 12406 (1991).

¹⁵ B. Hazell, H. Nevalainen, and P. Attfield, *FEBS Lett.* **377**, 457 (1995).

¹⁶ W. Boorstein and E. A. Craig, *Mol. Cell. Biol.* **10**, 3262 (1990).

¹⁷ A. Meyers, A. Tzagoloff, D. Kinney, and C. Lusty, *Gene* **299**, 299 (1986).

¹⁸ W. R. Boorstein and E. A. Craig, *EMBO J.* **9**, 2543 (1990).

increases to 61 units; continued growth until the diauxic shift is reached results in an increase to 290 units. Therefore, this promoter fusion provides an indication of both "heat shock" induction and glucose depletion.

RNA (Northern) and Immunoblots

The induction of heat shock genes can be monitored by direct detection of either the mRNA or the protein using standard methods. However, several things should be kept in mind when choosing tools for this analysis. Some of the heat-inducible proteins mentioned in the previous section are closely related to proteins that are constitutively expressed. Simple hybridization experiments will not distinguish between expression of these genes. The Hsp70 SSA family is particularly problematic in this regard, as *SSA2* and *SSA1* are expressed at high levels normally, whereas *SSA3* and *SSA4* are not. *HSP104* is a unique heat-inducible gene and is therefore more useful in simple hybridization experiments. However, it should be noted that expression of *HSP104* increases as cells approach stationary phase and is normally higher in cells grown on carbon sources other than glucose. *HSP26* is also a useful probe in hybridization experiments. Like *HSP104*, *HSP26* is a unique gene that is expressed at high levels on approach to stationary phase.¹⁹

Detection of the presence of heat shock proteins directly is the most accurate reflection of induction of the response because it measures the level of the proteins themselves. However, antibodies that react with most yeast heat shock proteins are not available commercially. An exception is Hsp104. Antibodies can be purchased from Affinity BioReagents, Inc. (Golden, CO) or StressGen Biotechnologies Corp. (Victoria, BC, Canada).

Importance of Interaction with Chaperone

Researchers most commonly come upon molecular chaperones in their work through either genetic or biochemical interactions. The challenge is to determine whether these interactions are biologically important. Typically, a biochemical interaction is found because of coimmunoprecipitation of a chaperone with the protein of interest. Genetic interactions can be found in synthetic lethal, multicopy suppressor or intragenic suppressor screens or selections. In our experience, the most productive way to test the importance of an interaction is to play genetics off of biochemistry and vice versa. If a biochemical interaction is found, use genetics to test its biological importance. If a genetic interaction is found, test for a direct biochemical interaction as well. Focusing on Hsp70 and Hsp90 interactions, we discuss ways to go about testing the nature of these interactions.

¹⁹ R. Susek and S. Lindquist, *Mol. Cell. Biol.* **10**, 6362 (1990).

Hsp70 Interactions

Hsp70s, found in several cellular compartments, are involved in many physiological functions, including protein folding, translocation of proteins into organelles, and assembly and disassembly of protein complexes. Hsp70s are often restricted to a particular cellular compartment, some to mitochondria, the endoplasmic reticulum (ER) lumen, or the cytosol and/or nucleus. All Hsp70s function with cochaperones called J-type chaperones (Hsp40s/DnaJs). At least 16 genes of the *S. cerevisiae* genome encode J-type chaperones. Some of these are experimentally well defined. In others cases it is only known that the encoded protein contains a signature "J" domain with the highly conserved HPD (histidine, proline, aspartic acid) motif. Fourteen members of the Hsp70 chaperone family are encoded in the *S. cerevisiae* genome. Therefore, it is impossible to comprehensively deal with individual Hsp70s in this chapter. Rather, we discuss methods that can be applied to the analysis of many different Hsp70s.

Hsp70 : Hsp40 Interactions. Hsp70s have an amino-terminal ATPase domain and a carboxy-terminal substrate-binding region. The interaction of Hsp70s with unfolded or partially unfolded polypeptides is regulated by ATP.³ Hsp40s bind the ATPase domain and, by stimulating ATP hydrolysis, promote the ADP-bound form of Hsp70, which has a higher affinity for unfolded protein substrates. All experiments to date indicate that Hsp70s always function together with cochaperones of the J class. Table I lists the Hsp70 : Hsp40 partnerships that are suggested by published data. Some of these partnerships, such as the Ssa Hsp70s and the J-type Ydj1 and Sis1 cochaperones of the cytosol, are well defined by both genetic and biochemical experiments. Others, such as the Ssb Hsp70s and the J-cochaperone Zuo1, are less well defined, based on genetic and *in vivo* colocalization studies. In individual cases the literature must be evaluated to determine the experimental foundation of the classification.

Analysis of Direct Interaction between Hsp70 and Protein of Interest. Coimmunoprecipitation of a molecular chaperone with the protein of interest leads to the question of the nature of the interaction and whether this interaction is important *in vivo*. Because of the propensity of Hsp70s to bind to exposed hydrophobic sequences in proteins, the first question is whether the interaction is occurring inside the cell or isolated organelle or whether the interaction occurs in the cell extract after lysis. This problem can be addressed by the addition of excess Hsp70 protein when lysing the cell/organelle, as has been done in the analysis of the mitochondrial Hsp70 Ssc1.^{20,21} If radiolabeled cells are being used, addition of an excess of unlabeled chaperone can be added prior to lysis. If binding

²⁰ J. Rassow, A. Maarse, E. Krainer, M. Kubrich, H. Muller, M. Meijer, E. Craig, and N. Pfanner, *J. Cell Biol.* **127**, 1547 (1994).

²¹ N. G. Kronidou, W. Opplinger, L. Bolliger, K. Hannavy, B. Glick, G. Schatz, and M. Horst, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12818 (1994).

TABLE I
Hsp70/Hsp40 CHAPERONE PAIRS

Site	Hsp70	Hsp40	Ref.
Cytoplasm	Ssa1-4	Ydj1 ^m	26
		Sis1	<i>a</i>
	Ssb1/2	Sis1	<i>b</i>
		Zuo1	<i>c</i>
		Zuo1	<i>d</i>
Endoplasmic reticulum	Sse1/2	?	<i>e</i>
		Sec63 ^m	<i>f</i>
	Kar2	Scj1	<i>g</i>
		Jem1	<i>h</i>
		Scj1	<i>i</i>
Mitochondria	Lhs1 (Cer1)	Scj1	<i>i</i>
	Ssc1	Mdj1 ^m	<i>j</i>
	Ssq1	Jac1	<i>k</i>
	Ecm10	?	<i>l</i>

^a Z. Lu and D. M. Cyr, *J. Biol. Chem.* **273**, 27824 (1998).

^b M. Ohba, *FEBS Lett.* **409**, 307 (1997).

^c W. Yan, B. Schilke, C. Pfund, W. Walter, S. Kim, and E. A. Craig, *EMBO J.* **17**, 4809 (1998).

^d T. Michimoto, T. Aoki, A. Toh-e, and Y. Kikuchi, *Gene* **257**, 131 (2000).

^e H. Mukai, T. Kuno, H. Tanaka, D. Hirata, T. Miyakawa, and C. Tanaka, *Gene* **132**, 57 (1993).

^f J. L. Brodsky and R. Schekman, *J. Cell Biol.* **123**, 1355 (1993).

^g G. Schlenstedt, S. Harris, B. Risse, R. Lill, and P. A. Silver, *J. Cell Biol.* **129**, 979 (1995).

^h V. Brizzio, W. Khalfan, D. Huddler, C. T. Beh, S. S. Andersen, M. Latterich, and M. D. Rose, *Mol. Biol. Cell* **10**, 609 (1999).

ⁱ T. G. Hamilton and G. C. Flynn, *J. Biol. Chem.* **271**, 30610 (1996).

^j B. Wettermann, B. Gaume, J. M. Herrmann, W. Neupert, and E. Schwarz, *Mol. Cell. Biol.* **16**, 7063 (1996).

^k C. Voisine, Y. C. Cheng, M. Ohlson, B. Schilke, K. Hoff, H. Beinert, J. Marszalek, and E. Craig, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1483 (2001).

^l F. Baumann, I. Milisav, W. Neupert, and J. M. Herrmann, *FEBS Lett.* **487**, 307 (2000).

^m Most thoroughly established Hsp70–Hsp40 interactions.

of the chaperone occurs during or after lysis, the amount of radiolabeled chaperone pulled down in the experiment will be decreased due to competition for binding with the unlabeled chaperone. However, if the interaction is occurring in the cell and is stable, addition of an exogenous chaperone should not affect the amount of radiolabeled chaperone coimmunoprecipitating with the protein of interest. Variations on this theme can also be used. For example, His-tagged chaperone can be added to cells on lysis, prior to immunoprecipitation. Assuming the His-tagged and untagged protein migrate differently in SDS–PAGE, the amount of binding by the native cellular chaperone in the presence or absence of an exogenously added His-tagged chaperone can be determined, without resorting to radiolabeling.

Interactions with Hsp70s can be divided into two categories: Hsp70 : substrate interactions and “typical” protein : protein interactions. Usually, a protein binding as a substrate (i.e., binding in the peptide-binding cleft) will be released on incubation with ATP, as binding of ATP increases the off rate of substrates dramatically (e.g., see Zhang *et al.*²²). Obviously, proteins may interact at sites other than the peptide-binding cleft. Such interactions may be independent of nucleotide, as is the interaction between the mammalian protein Hop and the extreme C terminus of Hsp70.²³ However, one such interaction that is disrupted by ATP is the interaction of the ATPase domain of the mitochondrial Hsp70 Ssc1 with the nucleotide exchange factor Mge1.²⁴ In addition, initiation of interactions between Hsp70s and J-type chaperones is dependent on ATP.²⁵

Finding the Meaning of Genetic Interactions with Hsp70s

In the case of genetic interactions, be it a synthetic lethal interaction of mutations or a suppression of mutant phenotypes, the question is whether there is direct involvement of the chaperone in the process being studied. Alternatively, the effect could very well be indirect, as by their nature increases or decreases in the activity of chaperones often have very pleiotropic effects. Strains carrying mutants in a number of HSP70 genes are available that allow testing to determine if a decrease in chaperone activity has an effect on the pathway or physiological process of interest. Table II lists some of the mutants that may be of use in such studies. Analysis of mutant SSA strains is complicated because Ssa proteins are encoded by four genes, at least one of which must be present for viability. Strains containing multiple mutations in genes encoding Ssa are available and have been used to demonstrate a role for Ssa in protein import into mitochondria, the ER, and vacuolar vesicles.^{26,27}

Hsp90 Interactions

Hsp90 is an essential, cytosolic chaperone accounting for 1–2% of all cytosolic proteins.²⁸ In *S. cerevisiae*, Hsp90 is encoded by two genes: the constitutively expressed *HSC82* and the heat-inducible *HSP82*. In mammalian cells, Hsp90 is critical for the activity of a number of signal-transducing proteins, such as steroid receptors, oncogenic tyrosine kinases, and additional diverse proteins involved in

²² S. Zhang, C. J. Williams, K. Hagan, and S. W. Peltz, *Mol. Cell. Biol.* **19**, 7568 (1999).

²³ J. Demand, J. Luders, and J. Hohfeld, *Mol. Cell. Biol.* **18**, 2023 (1998).

²⁴ B. Miao, J. E. Davis, and E. A. Craig, *J. Mol. Biol.* **265**, 541 (1997).

²⁵ A. K. Corsi and R. Schekman, *J. Cell Biol.* **137**, 1483 (1997).

²⁶ J. Becker, W. Walter, W. Yan, and E. A. Craig, *Mol. Cell. Biol.* **16**, 4378 (1996).

²⁷ C. R. Brown, J. A. McCann, and H. L. Chiang, *J. Cell Biol.* **150**, 65 (2000).

²⁸ K. A. Borkovich, F. W. Farrelly, D. B. Finkelstein, J. Taulien, and S. Lindquist, *Mol. Cell. Biol.* **9**, 3919 (1989).

TABLE II
Hsp70 MUTANT STRAINS

Site	Hsp70	Available mutants	Growth defects	Ref.
Cytoplasm	Ssa1-4	ssa1ssa2	Temperature sensitive	<i>a</i>
		ssa1 ⁴⁵ ssa2ssa3ssa4	Temperature sensitive	26
	Ssb1/2	Null	Cold sensitive	<i>b</i>
	Pdr13	Null	Cold sensitive	<i>c</i>
	Sse1	Null	Temperature sensitive	<i>d</i>
	Sse2	Null	None	<i>d</i>
Endoplasmic reticulum	Kar2	Conditional alleles	Temperature sensitive	<i>e, f</i>
	Lhs1	Null	Slightly cold sensitive	<i>g-i</i>
Mitochondria	Ssc1	Conditional alleles	Temperature sensitive	<i>j, k</i>
	Ssq1	Null	Cold sensitive	<i>l</i>
	Ecm10	Null	No reported defects	<i>m</i>

^a E. A. Craig and K. Jacobsen, *Cell* **38**, 841 (1984).

^b R. J. Nelson, T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne, and E. A. Craig, *Cell* **71**, 97 (1992).

^c T. C. Hallstrom, D. J. Katzmann, R. J. Torres, W. J. Sharp, and W. S. Moye-Rowley, *Mol. Cell. Biol.* **18**, 1147 (1998).

^d H. Mukai, T. Kuno, H. Tanaka, D. Hirata, T. Miyakawa, and C. Tanaka, *Gene* **132**, 57 (1993).

^e J. Polaina and J. Conde, *Mol. Gen. Genet.* **186**, 253 (1982).

^f J. P. Vogel, L. M. Misra, and M. D. Rose, *J. Cell Biol.* **110**, 1885 (1990).

^g R. A. Craven, M. Egerton, and C. J. Stirling, *EMBO J.* **15**, 2640 (1996).

^h T. G. Hamilton and G. C. Flynn, *J. Biol. Chem.* **271**, 30610 (1996).

ⁱ B. K. Baxter, P. James, T. Evans, and E. A. Craig, *Mol. Cell. Biol.* **16**, 6444 (1996).

^j P. J. Kang, J. Ostermann, J. Shilling, W. Neupert, E. A. Craig, and N. Pfanner, *Nature* **348**, 137 (1990).

^k B. D. Gambill, W. Voos, P. J. Kang, B. Miao, T. Langer, E. A. Craig, and N. Pfanner, *J. Cell Biol.* **123**, 109 (1993).

^l B. Schilke, J. Forster, J. Davis, P. James, W. Walter, S. Laloraya, J. Johnson, B. Miao, and E. Craig, *J. Cell Biol.* **134**, 603 (1996).

^m F. Baumann, I. Milisav, W. Neupert, and J. M. Herrmann, *FEBS Lett.* **487**, 307 (2000).

signaling pathways and the cell cycle control.^{7,29} Unlike Hsp70, which is believed to play a general role in chaperoning a wide range of cellular proteins, Hsp90 is probably not required for the bulk folding of cytosolic proteins,³⁰ rather it appears to be involved in the maturation of a diverse subset of proteins. In *S. cerevisiae*, Hsp90 interacts with heterologous substrates expressed in yeast and native substrates such as the MEK kinase Ste11,³¹ Gcn2, a member of the eIF-2 α kinase family,³² the

²⁹ W. B. Pratt and D. O. Toft, *Endocr. Rev.* **18**, 306 (1997).

³⁰ D. F. Nathan, M. H. Vos, and S. Lindquist, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 12949 (1997).

³¹ J. F. Louvion, T. Abbas-Terki, and D. Picard, *Mol. Biol. Cell* **9**, 3071 (1998).

³² O. Donze and D. Picard, *Mol. Cell. Biol.* **19**, 8422 (1999).

heme-responsive transcription factor Hap1,³³ the yeast heat shock factor Hsf1,³⁴ and Cna2, the catalytic subunit of calcineurin.³⁵

In yeast and mammalian systems, Hsp90 is found in a conserved complex with a number of cochaperones.^{29,36} In yeast, known Hsp90 cochaperones are Sti1, Sba1, Ydj1, Cdc37, Cns1, Cpr7, and Sse1.³⁷ Thus the appearance of Hsc82/Hsp82 or any of the cochaperones in a genetic or two-hybrid screen may indicate an interaction between the protein of interest and the Hsp90 complex. The following section outlines some approaches used to determine the significance of this interaction. As discussed earlier, a combination of genetic and biochemical approaches is most convincing, so if the interaction was first found genetically, determine a biochemical interaction, and viceversa. The three main approaches to determining a functional relationship between Hsp90 and a protein of interest are examining a direct interaction between Hsp90 and the protein of interest by coimmunoprecipitation or pulldown assays, determining the effect of pharmacological inhibition of Hsp90 function on the activity of the protein of interest, and assaying defects in substrate activity in the presence of mutations in Hsp90 or cochaperones.

Analysis of Direct Interaction between Hsp90 and Protein of Interest. Much of what is known about the interaction of Hsp90 with substrate proteins comes from the analysis of vertebrate steroid receptor complexes.²⁹ Hsp90 and cochaperones form stable complexes with receptors in the absence of hormone, when receptors are inactive as transcription factors. On hormone binding, Hsp90 and cochaperones dissociate, resulting in a transcriptionally active receptor. Hsp90 association is required for repression of receptor activity in the absence of hormone, as well as maintenance of the high-affinity hormone-binding state of the receptor. Hsp90 also has two roles in the maturation of Ste11, as Hsp90 is required for both repression of the pheromone pathway in the absence of pheromone and pheromone induction.³¹

Coimmunoprecipitation of Hsp90 with a protein of interest may be used to determine an Hsp90 interaction. Hsp90 interactions with substrate proteins are frequently stabilized in the presence of molybdate, albeit through an unknown mechanism.²⁹ Thus, the addition of 10 mM sodium molybdate to yeast lysis buffers may help stabilize the interaction between Hsp90 and the protein of interest. Generally, coprecipitation of Hsp90 with antibodies against a substrate protein is not difficult. However, because Hsp90 is very abundant, the level of any given substrate protein is likely much lower than the Hsp90 level, which may make detection of a coimmunoprecipitating substrate protein difficult. In some cases, overexpression of the substrate protein has been necessary for detection.³¹ An antibody to

³³ L. Zhang, A. Hach, and C. Wang, *Mol. Cell. Biol.* **18**, 3819 (1998).

³⁴ A. A. Duina, H. M. Kalton, and R. F. Gaber, *J. Biol. Chem.* **273**, 18974 (1998).

³⁵ J. Imai and I. Yahara, *Mol. Cell. Biol.* **20**, 9262 (2000).

³⁶ H. C. Chang and S. Lindquist, *J. Biol. Chem.* **269**, 24983 (1994).

³⁷ A. J. Caplan, *Trends Cell Biol.* **9**, 262 (1999).

yeast hsc82/hsp82 is not available commercially. However, investigators have successfully coimmunoprecipitated Hsp90–substrate complexes using Hsc82/Hsp82 antibodies³⁵ or Flag-tagged Hsc82.³¹

Pharmacological Inhibition of Hsp90 Interaction. Once a direct interaction between Hsp90 and the protein of interest has been observed, the challenge is to determine whether the interaction is specific and has functional consequences. Hsp90 has ATPase activity that is essential for its *in vivo* function. However, the interaction between Hsp90 and substrates is complex, and the interaction may not be monitored by binding in the presence or absence of ATP, as for Hsp70.^{6,7} However, Hsp90 may be inhibited *in vitro* and *in vivo* with the ansamycin antibiotics geldanamycin (GA) and macbecin I. These drugs are available through the Developmental Therapeutics Program at the National Cancer Institute (Bethesda, MD). GA has been found to specifically decrease the activity of a number of mammalian Hsp90–substrate proteins, such as steroid receptors and oncogenic tyrosine kinases. The related drug, macbecin I, is more effective than GA at inhibition of *in vivo* Hsp90 activity in *S. cerevisiae*.³⁸ As discussed later, these drugs may be used to establish the functional importance of the interaction between substrate proteins and Hsp90 *in vitro* and *in vivo*.

Conservation of Hsp90 function between mammalian and yeast systems allows functional analysis of heterologous substrates expressed in yeast Hsp90, as well as yeast proteins expressed in rabbit reticulocyte lysates. GA is particularly useful when the protein of interest has an activity that may be assayed after transcription and translation in reticulocyte lysate. For example, the synthesis of Gcn2 in reticulocyte lysates in the presence of GA resulted in a dramatic reduction in *in vitro* kinase activity, helping to demonstrate a role for Hsp90 in kinase maturation. In addition, GA treatment of reticulocyte lysate resulted in an increased Hsp90–Gcn2 interaction, possibly by inhibiting the release of Hsp90 required for kinase activity.³² This result, which contrasts with studies in which GA treatment decreases Hsp90 interaction with steroid receptors,²⁹ is likely an indication of the many ways in which Hsp90 association may affect substrate activity.

Geldanamycin and the related drug macbecin I may also be used to inhibit Hsp90 activity *in vivo*. The role of Hsp90 in substrate maturation may then be assayed by the treatment of cells with macbesin I prior to coimmunoprecipitation or enzymatic assay. A caveat of using these drugs to inhibit Hsp90 association is that the level of the substrate protein is frequently decreased in the absence of Hsp90 interaction, presumably due to the intrinsic instability of many Hsp90 substrates in the absence of Hsp90 or their respective ligands. For example, the *in vivo* levels of Gcn2 or Cna2 decreased on treatment with macbesin I or GA.^{32,35}

Mutations in Hsp90 and Cochaperones. Mutations in Hsp90 and cochaperones have been found to specifically affect the activity of heterologous steroid receptors

³⁸ S. P. Bohen, *Mol. Cell. Biol.* **18**, 3330 (1998).

TABLE III
Hsp90 AND COCHAPERONE MUTANT STRAINS

Protein	Available mutants	Growth defects	Ref.
Hsc82/Hsp82	Reduced level	Temperature sensitive	39
	Reduced activity	Temperature sensitive	40 ^{a,b}
	Conditional alleles	Temperature sensitive	40
Sba1	Null	None	38 ^c
Sti1	Null	Temperature sensitive	<i>d</i>
Sse1	Null	Temperature sensitive	<i>e, f</i>
Cpr7	Null	Slow growth	<i>g</i>
Cdc37	Conditional alleles	Temperature sensitive	<i>h-j</i>
Ydj1	Null, conditional alleles	Temperature sensitive	<i>k, l</i>

^a Y. Kimura, S. Matsumoto, and I. Yahara, *Mol. Gen. Genet.* **242**, 517 (1994).

^b S. P. Bohen and K. R. Yamamoto, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 11424 (1993).

^c Y. Fang, A. E. Fliss, J. Rao, and A. J. Caplan, *Mol. Cell. Biol.* **18**, 3727 (1998).

^d C. Nicolet and E. Craig, *Mol. Cell. Biol.* **9**, 3638 (1989).

^e X. D. Liu, K. A. Morano, and D. J. Thiele, *J. Biol. Chem.* **274**, 26654 (1999).

^f H. Mukai, T. Kuno, H. Tanaka, D. Hirata, T. Miyakawa, and C. Tanaka, *Gene* **132**, 57 (1993).

^g A. A. Duina, J. A. Marsh, and R. F. Gaber, *Yeast* **12**, 943 (1996).

^h B. Dey, J. J. Lightbody, and F. Boschelli, *Mol. Biol. Cell.* **7**, 1405 (1996).

ⁱ M. R. Gerber, A. Farrell, R. J. Deshaies, I. Herskowitz, and D. O. Morgan, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 4651 (1995).

^j S. I. Reed, *Genetics* **95**, 561 (1980).

^k J. L. Johnson and E. A. Craig, *Mol. Cell. Biol.* **20**, 3027 (2000).

^l B. Dey, A. J. Caplan, and F. Boschelli, *Mol. Biol. Cell* **7**, 91 (1996).

and v-src expressed in yeast. Now that the effect of these mutations on known Hsp90 substrates has been demonstrated, they become valuable tools in helping identify novel Hsp90 substrates. Table III lists some of the mutant strains that have been shown to affect the activity of Hsp90 substrates.

Assays of Substrate Activity in Hsp90 Mutant Cells. Hsp90 is essential in yeast, and the available *hsc82 hsp82* mutant yeast strains exhibit either reduced Hsp90 activity at all temperatures or temperature-sensitive activity. The most commonly used mutant Hsp90 yeast strain, GRS4, contains chromosomal deletions of *hsc82* and *hsp82* in combination with a plasmid-borne copy of *HSP82* under the *GALI* promoter. Wild-type levels of Hsp90 are produced when cells are grown in the presence of galactose. However, in the presence of glucose, the leaky promoter results in expression of 5–10% of the wild-type level of Hsp90.³⁹ This strain was used to demonstrate a role for Hsp90 in Hap1 activation, as Hap1 activity in the presence of glucose was sharply reduced relative to that observed in the

³⁹ D. Picard, B. Khursheed, M. Garabedian, M. Fortin, S. Lindquist, and K. Yamamoto, *Nature* **348**, 166 (1990).

presence of galactose.³³ Although the level of Hsp1 was unaffected, the levels of Cna2 were dramatically decreased in cells grown in glucose,³⁵ indicating that the stability of some Hsp90 substrates may be affected by decreased levels of Hsp90.

Hsp82 point mutants have been isolated in three independent genetic screens (see Table III). These mutants were generated in *hsc82 hsp82* disruption strains containing mutagenized *HSP82*. Most of these mutants exhibit reduced Hsp90 activity at 25°. Thus, the activity of Hsp90 substrates, such as GCN2, is decreased in strains expressing these mutants. Another tool for analyzing the role of Hsp90 is use of the conditional temperature-sensitive allele G170D.⁴⁰ While strains expressing this mutant behave like wild type at 25°, the protein rapidly becomes inactive at 37° (within 90 min), resulting in loss of Hsp90 function. This mutant was used to show that loss of Hsp90 function has a dramatic effect on the repression of Hsf1.³⁴

Evidence that Hsp90 plays a role in the maturation of a protein of interest is further supported when Hsp90 cochaperones are also shown to be required. The observation that Gcn2 activity decreased in strains containing mutations in *STII*, *CDC37*, and *SBA1* was used to help establish a role for Hsp90 in kinase maturation.³² Table III lists many of the available mutations in Hsp90 cochaperones. The Hsp90 cochaperones Sba1(p23), Sti1 (Hop), Cpr7 (Cyp-40), Ydj1 (Hsp40), and Sse1 (a member of the Hsp110 family) are encoded by single nonessential genes. Cdc37, which may be associated with only a subset of Hsp90 substrates,⁴¹ is encoded by an essential gene. Although it may be possible that cochaperones have cellular functions independent of Hsp90 function, the involvement of Hsp90 cochaperones in a particular cellular process provides valuable evidence that Hsp90 is also involved in that process.

⁴⁰ D. F. Nathan and S. Lindquist, *Mol. Cell. Biol.* **15**, 3917 (1995).

⁴¹ J. Rao, P. Lee, S. Benzeno, C. Cardozo, J. Albertus, D. M. Robins, and A. J. Caplan, *J. Biol. Chem.* **276**, 5814 (2001).