

A. Work Accomplished by You and/or Others

Cellular biology of protein chaperones

Protein molecular chaperones play critical roles in cellular biology. The family of proteins classified as molecular chaperones is large and diverse, but is united by a common characteristic – the ability to form transient yet productive interactions with a broad array of substrate proteins and foster acquisition of a functional form (reviewed in Buchner, 1996). For many proteins, it is a “cradle to grave” interaction, with chaperones binding early upon emergence from the ribosome (Pfund et al., 1998), maintenance of a persistent substrate-chaperone heterocomplex (Pratt and Toft, 1997), and in some cases directed delivery of the protein to the proteolytic degradation machinery (Schneider et al., 1996). While the biochemistry of these interactions has been the subject of many years of productive investigation, how the cell regulates chaperone production and function, and integrates chaperones into cellular processes during both stress and non-stress conditions, is not well understood. Protein chaperones are perhaps best known for their roles in protein biogenesis, particularly as required cofactors for proteins which must traverse one or more biological membranes in an unfolded state prior to full maturation (reviewed in Craig et al., 1994). In addition, much attention has been focused on subclasses of chaperones that partially unfold and then refold denatured, aggregated, unfolded, or otherwise damaged proteins, frequently after severe proteotoxic stress. Accordingly, cells lacking these chaperones are severely compromised in thermotolerance, the ability to withstand short periods of intense stress (Sanchez and Lindquist, 1990). Only recently has another role for protein chaperones come under scrutiny, centered on one key player, the highly conserved and ubiquitous chaperone Hsp90.

transcription factors	kinases
steroid hormone receptors	v-Src, c-Src
heat shock factor/HSF1	Raf/Ste11
mutant p53 tumor suppressor	Gcn2
MyoD	calcineurin
hypoxia-inducible factor	CDK/Cdc28
ecdysone receptor	eIF-2 α kinase
Hap1	Wee1
other	Lck
nitric oxide synthase	casein kinase-II
reverse transcriptase	c-Mos
telomerase	MEK

The Hsp90 chaperone complex

In contrast to nearly all of the other chaperone families, Hsp90 does not function as a general chaperone and is not required for thermotolerance (Nathan et al., 1997). Instead, Hsp90 exhibits a high degree of selectivity for a small but growing number of so-called “client” proteins. These substrates, while diverse, share a common theme of being involved in critical processes in cell growth, differentiation and signaling, and include transcription factors, protein kinases and signaling enzymes such as nitric oxide synthase (see Fig. 1 for an abridged list of known clients, reviewed in (Mayer and Bukau, 1999; Pratt, 1998)). Moreover, all Hsp90 clients display a marked dependency on the chaperone for both stability and activity even under non-stress conditions, and

Fig.1 Clients of the Hsp90 chaperone complex. A sampling of known Hsp90-dependent proteins from yeast and higher eukaryotes. A number of these proteins play roles in human cancer.

consequently high levels of Hsp90 are found in all eukaryotic cells. A hallmark of Hsp90-client protein interactions is that client proteins remain tightly associated with the chaperone in an inactive but activation-competent state, and upon activation by ligand association or some other post-translational regulatory event, are released as stable, functional molecules. For example, the classic model of Hsp90-dependent signaling is the steroid hormone receptor family, members of which require interaction with Hsp90 and additional co-chaperones to fold into a hormone binding-competent state (Pratt, 1997). Upon steroid binding, the receptor is released in a transcriptionally active form. Likewise, both the oncogenic tyrosine protein kinase v-src^{pp60} (Xu and Lindquist, 1993) and the cell cycle kinase Cdk4 (Stepanova et al., 1996)

exhibit an absolute dependence on Hsp90 for catalytic activity through upstream activation or cyclin association, respectively. Many Hsp90 clients are isolated from cell extracts in a number of discrete chaperone heterocomplexes. A great deal of biochemical investigation coupled with the use of genetics in both *S. cerevisiae* and *Drosophila* has elucidated a chaperone folding cycle which can involve up to twelve chaperones and co-chaperones associating with a client protein prior to final activation, as listed in Fig. 2 (reviewed in Caplan, 1999). The roles of many of these chaperones in client protein folding are unclear, but nearly all are highly conserved from yeast to man, and are functionally interchangeable between systems. Two additional components of the Hsp90 chaperone system have been described thus far only in yeast: Cns1, a protein containing multiple copies of the tetratricopeptide repeat (TPR) motif (Dolinski et al., 1998; Marsh et al., 1998), and Sse1, one of two yeast homologs of the Hsp70-related Hsp110 family of protein chaperones (Liu et al., 1999; Mukai et al., 1993). This latter chaperone family is well-represented in mammalian genomes, with multiple highly expressed tissue-specific isoforms of unknown function (Lee-Yoon et al., 1995). The biomedical importance of most of the proteins in the Hsp90 client catalog, especially those intimately involved in oncogenesis, underscores the need to understand in detail the roles the chaperone plays in their maturation and function. Additionally, the involvement of Hsp90 in a number of key cellular signaling networks provides an attractive nexus of regulation – by raising or lowering the “chaperone capacity” of the Hsp90 system, the cell could rheostatically control a number of signaling

Higher Eukaryotic Chaperone	Yeast Ortholog	Function
Hsp90	Hsp82, Hsc82	chaperone
Hsp70	SSA1-4	chaperone
Hsp40	Ydj1	Hsp70 regulator
HOP	Sti1	mediates Hsp90-Hsp70 binding
BAG-1	no	Hsp70 regulator
Hip	no	Hsp70 regulator
CHIP	no	protein triage factor
Cyp-40	Cpr6, Cpr7	peptide-prolyl isomerase
p23	Sba1	stabilizes Hsp90 substrate
p50	Cdc37	chaperone
no	Cns1	unknown; binds Hsp90
Hsp110	Sse1, Sse2	chaperone

Fig. 2 Hsp90 and its co-chaperones

functions in the cell, many still poorly characterized (reviewed in Thevelein and de Winde, 1999). While the mechanism by which Ras senses optimal growth conditions to maintain high PKA activity is unclear, high glucose is thought to be the primary activator and the entire system is finely tuned to respond to this carbon source. In higher eukaryotes, adenylate cyclase regulation is primarily under the control of heterotrimeric GTP binding proteins, and it was only very recently that a similar Gα/G-protein coupled receptor pair, Gpa2/Gpr1, was identified and characterized in yeast (Colombo et al., 1998; Xue et al., 1998). Thus, parallel systems operate to sense and transduce available levels of glucose, and perhaps other nutrients as well. Similarly, a number of lines of evidence support a model whereby the Sch9 kinase functions in parallel with the cAMP-PKA pathway. First, overexpression of the *SCH9* gene allows growth

pathways in response to environmental conditions. The recent identification of the serine/threonine protein kinase Sch9 as a regulator of Hsp90 signaling functions in yeast strongly supports this hypothesis and forms the cornerstone of this proposal (Morano and Thiele, 1999b).

Sch9 as a growth control kinase

The Sch9 kinase was first isolated as a high-copy suppressor of a temperature sensitive allele of the Ras-activating gene *CDC25* (Toda et al., 1988). In yeast, the small GTPase Ras is a key activator of adenylate cyclase, the enzyme responsible for production of cAMP, which in turn binds the regulatory subunit of protein kinase A (PKA), Bcy1 (Broach and Deschenes, 1990). Dissociation of Bcy1 complexed with cAMP activates PKA, which carries out a myriad of growth control

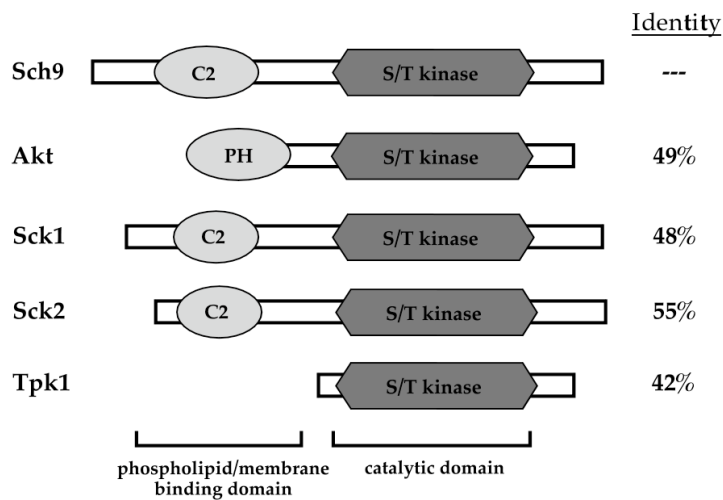


Fig. 3 Alignment of Sch9 and homologs. Human Akt kinase, the *S. pombe* Sck1 and Sck2 kinases and the PKA catalytic subunit Tpk1 from *S. cerevisiae*. PH: pleckstrin homology domain, S/T: serine/threonine.

in mutant strains in which all three of the collectively essential PKA catalytic subunits are eliminated (Toda et al., 1988). Second, *ras2Δ* and *sch9Δ* mutations exhibit a synthetic interaction, displaying an additive growth defect greater than that observed in either mutant strain alone (Lorenz et al., 2000). Third, *sch9Δ* mutant cells exhibit a pronounced slow-growth phenotype despite normal levels and function of the cAMP-PKA pathway, indicating that the PKA and Sch9 kinases have distinct roles in growth control (Toda et al., 1988). The Sch9 kinase is highly similar to two likely orthologs in the fission yeast *Schizosaccharomyces pombe*, and most homologous to the growth control kinase Akt (protein kinase B) in mammalian cells, as depicted in Fig. 3 (Bellacosa et al., 1991; Fujita and Yamamoto, 1998; Jin et al., 1995).

Akt is a major effector of growth and survival factor signaling through the phosphoinositide-3 (PI3) kinase pathway, is required for glucose uptake and is a major inhibitor of apoptosis (reviewed in Marte and Downward, 1997). Moreover, Akt may contribute to neoplastic transformation, as it is overexpressed in a variety of carcinomas (Bellacosa et al., 1995; Cheng et al., 1992). The amino-terminus of Akt contains a pleckstrin-homology (PH) domain essential for membrane localization and phosphoinositide signaling (Franke et al., 1997). While signaling through PI3 has not been demonstrated in yeast, both the *S. cerevisiae* and *S. pombe* homologs contain a C2 domain, which has been implicated in phospholipid binding and membrane targeting in other proteins such as the protein kinase C family (Nalefski and Falke, 1996). It is possible that the PH and C2 domains may play analogous regulatory roles in the biology of Akt and Sch9, respectively. Importantly, overexpression of Akt represses glucocorticoid receptor activity in mammalian cells (Liao et al., 1998), which would be predicted if Akt influences mammalian Hsp90 activity as Sch9 does in yeast.

A truncation mutant of yeast HSF is defective in Hsp90 signaling

Basal and stress-induced expression of heat shock proteins (Hsps) is governed by the **heat shock transcription factor** (HSF) in all eukaryotic cells. HSF binds as a homotrimer to cognate DNA sequences called heat shock elements, which lie upstream of heat shock genes (reviewed in Morimoto et al., 1996). In many metazoan cell types, multiple HSF isoforms differentially regulate HSP expression in response to stress, cell differentiation and developmental signals (reviewed in Morano and Thiele, 1999a). In yeast, however, HSF is encoded by a single, essential locus and activates distinct subsets of heat shock genes in response to environmental insults such as heat shock and oxidative stress (Nieto-Sotelo et al., 1990; Sorger and Pelham, 1987). Yeast HSF also contains two separate transcriptional activation domains, located at the amino and carboxyl termini of the protein; deletion of the C-terminal domain (HSF(1-583)) results in a temperature sensitive growth phenotype without affecting cell growth at normal temperature (Sorger, 1990). I further investigated this phenotype and found that HSF(1-583) cells arrest in the G2/M phase of the cell cycle, one of the first demonstrated links between HSF function and cell proliferation (Morano et al., 1999). To determine the molecular nature of this phenotype, I performed a dosage dependent suppressor

screen using a high-copy yeast genomic library. I isolated one of the two isoforms of yeast Hsp90, the heat shock-inducible *HSP82* (Morano et al., 1999). I was further able to demonstrate that HSF(1-583) cells are in fact deficient in Hsp90 chaperone function due to greatly reduced levels of Hsp90 and the other heat inducible co-chaperones (Liu et al., 1999). This diminished expression renders HSF(1-583) cells susceptible to Hsp90 inhibitors such as geldanamycin, an antitumor drug currently in clinical trials at the National Cancer Institute. Specific depletion of Hsp90 from wild type cells also results in cell cycle arrest, demonstrating a requirement for Hsp90 for cells to proliferate under heat stress (Morano et al., 1999). Based on its role as a powerful facilitator of signal transduction, I reasoned that the function of Hsp90 might itself be regulated by the cell to achieve rheostatic control over various aspects of signaling and physiology. **Because the HSF(1-583) mutation renders cells defective for Hsp90 function by coordinately reducing expression of Hsp90 and many of its co-chaperones, it is an ideal tool to use for genetic suppression analysis** – no individual chaperone is completely eliminated, as would be the case using null alleles, nor are there inactivating or otherwise hypomorphic mutations in the amino acid sequences.

Sch9 is a regulator of Hsp90 chaperone activity

I utilized the temperature sensitive phenotype of this strain to isolate suppressors of the cell cycle arrest, which might be expected to regulate Hsp90 activity. One such suppressor encoded a loss of function allele of the previously introduced serine/threonine protein kinase *SCH9* (Morano and Thiele, 1999b). Loss of the Sch9 kinase results in a striking restoration of Hsp90 signaling in the HSF(1-583) mutant, as assayed by multiple Hsp90-dependent target systems, indicating that Sch9 negatively regulates Hsp90 chaperone complex function ((Morano and Thiele, 1999b); see Fig. 4). In addition, because Hsp90 modulates the function of the mitogen activated protein (MAP) kinase-kinase-kinase Ste11, loss of Sch9 in wild type cells also results in activation of the pheromone-response MAP kinase cascade in the absence of pheromone, suggesting that Hsp90 activity must be controlled to avoid hyperactivation of Hsp90-dependent client proteins (Morano and Thiele, 1999b). Importantly, steady-state levels of Hsp90 and its co-chaperones are

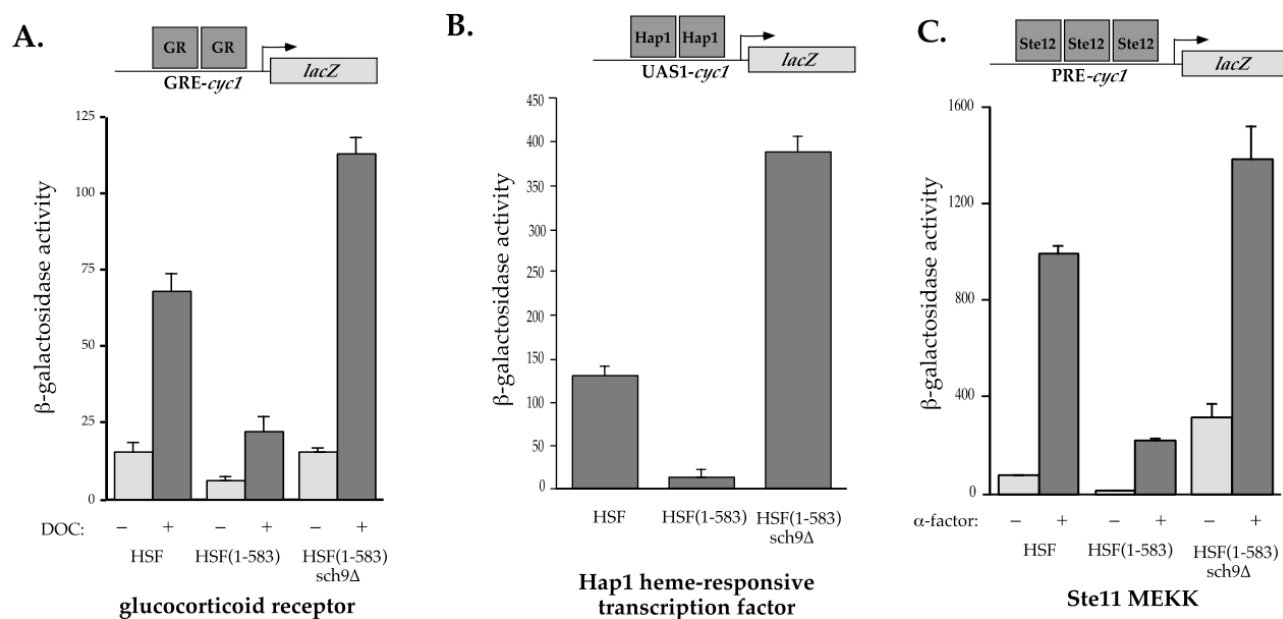


Fig. 4 Derepression of Hsp90 chaperone activity in HSF(1-583) *sch9Δ* cells assayed on three different *in vivo* substrates. At the top of each graph is shown the relevant β-galactosidase reporter. DOC: deoxycorticosterone, activator of GR, α-factor: activator of yeast pheromone response pathway.

NOT increased upon deletion of *SCH9* (Morano and Thiele, 1999b). Therefore restoration of Hsp90-dependent signaling must be due to increased chaperone function from the Hsp90 complex. Loss of the Sch9 kinase clearly restores Hsp90 signaling in the HSF(1-583) mutant background as measured by the “terminal” readout of transcriptional activation via the heterologously expressed rat glucocorticoid receptor (GR), but what is the molecular nature of this effect? To gain additional mechanistic data, the steady-state levels of a functional GR tagged with the FLAG epitope (Chang and Lindquist, 1994) were examined via immunoblot of cell extracts. Many Hsp90 client proteins, including GR, are destabilized *in vivo* when Hsp90 function is compromised. As seen in Fig. 5, the steady state levels of FLAG-GR were substantially reduced in the HSF(1-583) mutant, consistent with the previously observed diminished transcriptional activity. Elimination of the Sch9 kinase increased FLAG-GR protein levels, indicative of a more

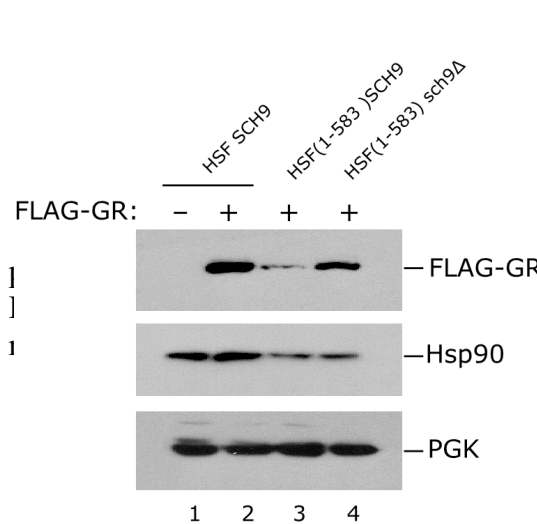


Fig. 5 Loss of Sch9 stabilizes an Hsp90 client protein in HSF(1-583) cells. The indicated strains were transformed with a plasmid expressing FLAG-GR, or an empty vector control. Protein extracts were made from cells grown under standard conditions, resolved by SDS-PAGE, and immunoblotted with the indicated antibodies. PGK: phosphoglycerate kinase, used for load control.

To confirm that the changes in GR levels are due to differential protein stability rather than synthesis, a pulse-chase radiolabeling experiment was performed, shown in Fig. 6.

In keeping with the diminished steady-state levels of GR as determined by immunoblot analysis, the half-life of radiolabeled GR was dramatically reduced in the HSF(1-583) mutant, and partially restored by deletion of the *SCH9* gene. Together these data demonstrate a role for Sch9 in Hsp90 chaperone activity at the molecular level, using a benchmark substrate. What then is the mechanism of Sch9 regulation of Hsp90 function? Preliminary results suggest that Hsp90 itself is not likely to be the direct target of phosphorylation, as shown in Fig. 7. FLAG-Hsp90 immunoprecipitated from both wild type and *sch9Δ* cells labeled with radioactive inorganic phosphate displayed nearly identical intensities of labeling. This indicates that like mammalian Hsp90, yeast Hsp90 is a phosphoprotein

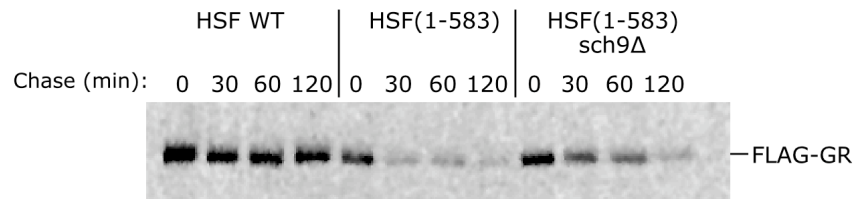


Fig. 6 Loss of Sch9 increases the half-life of an Hsp90 client. The indicated strains transformed with the FLAG-GR plasmid were subject to pulse-chase radiolabeling with Met-S³⁵, followed by immunoprecipitation with anti-FLAG antibody.

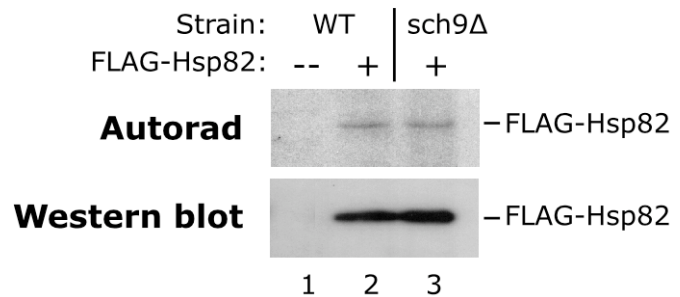


Fig. 7 Hsp90 phosphorylation is not diminished in an sch9Δ strain. The indicated strains transformed with a plasmid expressing a FLAG-tagged yeast Hsp90 (Hsp82) were radiolabeled with PO₄³², followed by immunoprecipitation with anti-FLAG antibody. Parallel nitrocellulose blots were read by PhosphorImager analysis or immunoblotted with anti-FLAG antibody to confirm recovery and loading.

(Legagneux et al., 1991; Mimnaugh et al., 1995), and that the Sch9 kinase likely does not provide a major contribution. Together, these preliminary data strongly support a model wherein the Sch9 kinase is directly regulating a modulator of Hsp90 complex function which in turn has a profound effect on Hsp90-dependent signal transduction activities in vivo. The experiments in specific aim 1 are directed toward identifying this protein, characterizing its regulation by Sch9, and elucidating its roles in Hsp90 function.

The ras-cAMP-PKA may participate in Hsp90 regulation

The Sch9 kinase is closely related to the PKA family of growth control kinases. Genetic epistasis experiments suggest that the two kinase families operate largely independently, but may share some common substrates. Because overexpression of either kinase suppresses phenotypes arising from elimination of the other, the kinases are likely to have highly similar phosphorylation consensus sites. Importantly, no consensus site for phosphorylation by Sch9 has been determined. In fact, while ample evidence exists for Sch9 functioning as a protein kinase in vivo (Crauwels et al., 1997; Morano and Thiele, 1999b; Toda et al., 1988), there is to date no published demonstration of in vitro kinase activity. I therefore explored the possibility that known components of the ras-cAMP pathway might also be involved in Hsp90 regulation. The yeast analog of the oncogenic Ras allele Ras^{val12}, which results in transformation of human cell lines, functions similarly in yeast by continuing to promote growth in spite of nutrient depletion, leading to an inability to acquire protective characteristics associated with the starvation response (DeFeo-Jones et al., 1983; Kataoka et al., 1985). Introduction of this allele (Ras2^{val19}) into HSF(1-583) cells results in drastic growth impairment in a manner similar to that obtained by overexpression of the Sch9 kinase, consistent with increased inhibition of the Hsp90 chaperone complex, leading to cell death (Fig. 8).

In yeast, three independent genes, *TPK1*, *TPK2* and *TPK3*, encode catalytic subunits of protein kinase A. Deletion of any two genes has no effect on cell viability, but elimination of all three genes is lethal (Toda et al., 1987). A classic mutant with reduced PKA activity is the so-called *tpk^w* or “wimp” strain, in which two of the *TPK* genes, as well as the *BCY1* regulatory subunit, are deleted, and one *TPK* allele contains uncharacterized hypomorphic mutations (Cameron et al., 1988). This mutant displays all the characteristics of low Ras-cAMP pathway activity, including increased glycogen accumulation and increased resistance to heat shock (Cameron et al., 1988).

To ascertain whether PKA activity contributes to Hsp90 regulation in parallel with Sch9, isogenic parent and *tpk^w*

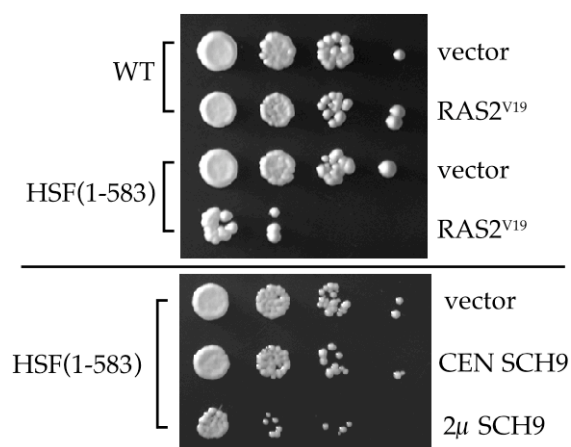


Fig. 8 Overexpression of the activated RAS2^{val19} allele mimics the growth inhibition of HSF(1-583) cells caused by SCH9 overexpression. The indicated strains were transformed with a plasmid expressing the activated Ras allele or vector alone, or the indicated low or high-copy SCH9 plasmids. Cultures of equal density were spotted in a dilution series on selective plates.

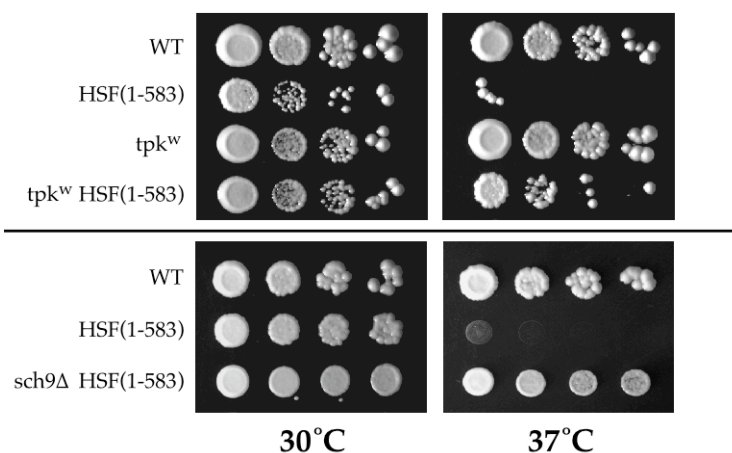


Fig. 9 Reduction of cellular PKA activity mimics sch9Δ. Cultures of equal density were spotted in a dilution series on selective plates. All four strains in the upper panel are in the SP1 background, the bottom three strains are in the W303 background.

strains were mutagenized by replacement of the wild type HSF locus with the HSF(1-583) allele. As shown in Fig. 9, lowered PKA activity partially suppresses the HSF(1-583) cell cycle arrest/temperature sensitivity phenotype, albeit to a lesser extent than *sch9Δ*. Together, these data suggest that both the Sch9 and PKA kinases contribute to negative regulation of Hsp90 chaperone function, and that elimination of either one is sufficient to partially alleviate this repression.

Importantly, recent analyses of Ras-cAMP signaling demonstrate a synthetic phenotype between *ras2Δ* and *sch9Δ* mutations, or *gpa2Δ* and *sch9Δ* mutations, reinforcing the idea that these two systems perform independent functions in vivo (Kraakman et al., 1999; Lorenz et al., 2000). Regulation of Hsp90 by one or both of these systems may also be responsive to nutrient levels. It is well known that PKA activity is greatest in glucose-grown cells (reviewed in Thevelein et al., 2000), and a recent report suggests that the Gpr1-Gpa2 G-protein-coupled-receptor system may also sense nitrogen levels (Xue et al., 1998). If Sch9/PKA inhibition of Hsp90 function is indeed responsive to cellular nutrient status, then growth on a less favorable carbon source may lead to diminished kinase activity, thereby derepressing Hsp90 and allowing growth of HSF(1-583) cells at 37°C. This was indeed found to be the case, as shown in Fig. 10. HSF(1-583) cells plated on 2% glucose minimal medium are unable to grow at 37°C, while the same strain plated on 2% galactose is viable. These data support a model whereby nutrient levels are sensed by the growth control kinases Sch9 and/or PKA, leading to derepression of chaperone activity under non-optimal growth conditions.

The Sse1 molecular chaperone, Hsp90 and regulation by Sch9

While a postdoctoral researcher in the laboratory of Dennis Thiele, University of Michigan, a graduate student in the lab and I found that the yeast Hsp110 family member, Sse1 (Mukai et al., 1993), participated in Hsp90 function. Sse1 was shown to be part of a multichaperone complex with Hsp90, and *SSE1* gene deletions exhibited synthetic interactions with deletion of other Hsp90 co-chaperones, as well as the HSF(1-583) allele (Liu et al., 1999). Little is known about this chaperone family in yeast or higher eukaryotes, and this was the first demonstration of a potential functional role. The stoichiometric balance of Sse1 with the other chaperones is critical, as overexpression of Sse1 is also detrimental to HSF(1-583) cells, which express lower levels of Hsp90, Sti1, Cpr6 and Ydj1, demonstrated in Fig. 11. This effect must be post-translational, as cells overexpressing Sse1 show no diminution in HSF-mediated chaperone expression after heat shock (unpublished data). With the goal of determining the chaperone requirements for Sch9 regulation, I recently constructed a number of strains carrying *sch9Δ* deletions and deletions of the various Hsp90 co-chaperones. Although most of the allele combinations display no significant or unusual phenotypes, the *sse1Δ sch9Δ* double mutant strain exhibits a very pronounced temperature-conditional synthetic phenotype at 37°C, as shown in Fig. 12. This suggests that the cellular roles of Sse1 and Sch9 may be functionally related and underscore the importance of determining the roles of these two proteins in Hsp90 regulation and function.

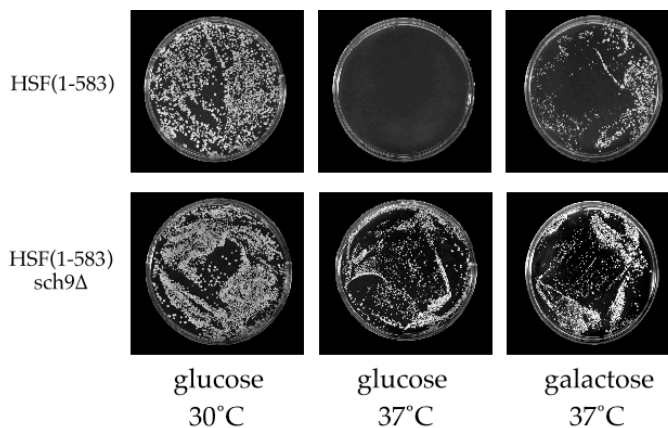


Fig. 10 Growth on non-optimal carbon sources partially suppress HSF(1-583) *ts* phenotype. Cultures of equal density were diluted and spread on selective plates, followed by growth at the indicated temperatures. Colonies from the HSF(1-583) strain grown at 37°C were less robust than HSF(1-583) *sch9Δ* cells grown at the same temperature on glucose.

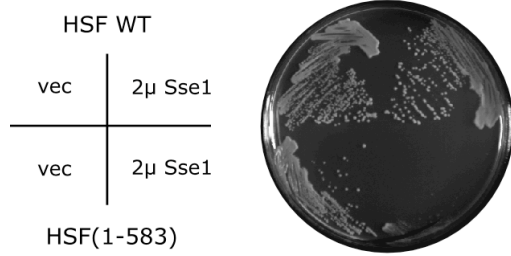


Fig. 11 Overexpression of *SSE1* is synthetically lethal with HSF(1-583) at 30°C. The indicated strains were transformed with vector alone (vec) or a high-copy plasmid overexpressing the *SSE1* chaperone and grown at 30°C for 3 days.

A working model for regulation of the Hsp90 chaperone complex by the Sch9 and PKA kinases is shown in Fig. 13. In this model, the activities of both kinases are modulated by environmental conditions such as nutrient status, with maximal activity occurring during nutrient replete conditions. Hsp90 activity is repressed under these conditions by an unknown mechanism, perhaps generating an inactive or partially active pool of chaperones. Upon reduction of Sch9 and/or PKA activity, this repression is relieved and additional Hsp90 stores are mobilized to provide the necessary chaperone activity for cellular defense and metabolic reprogramming. The functions of the few Hsp90 targets known in yeast support this conjecture: the Gcn2 kinase is required for activation of the yeast transcription factor Gcn4 upon amino acid starvation (Dever et al., 1992), the Ste11 kinase is involved in pseudohyphal differentiation on nitrogen-limiting media (Roberts and Fink, 1994), and the Hap1 transcription factor promotes expression of genes required for respiration (Creusot et al., 1988; Pfeifer et al., 1987).

Fig.13 Conceptual model for cellular regulation of the Hsp90 complex by Sch9 and the cAMP-PKA pathway. The yeast Hsp90 chaperone complex, composed of the indicated subunits, is required for the synthesis, stability and function of a variety of cellular targets essential for the cellular processes shown in boxes at bottom. An unknown factor, indicated with question marks, is hypothesized to mediate inhibition of the Hsp90 complex by Sch9 and the PKA pathway. Sch9 is hypothesized to exist in both membrane-associated and soluble cytosolic pools, the distribution of which may depend upon signaling inputs. Two parallel pathways for activating adenylate cyclase, and therefore PKA, are indicated and will be examined in this proposal.

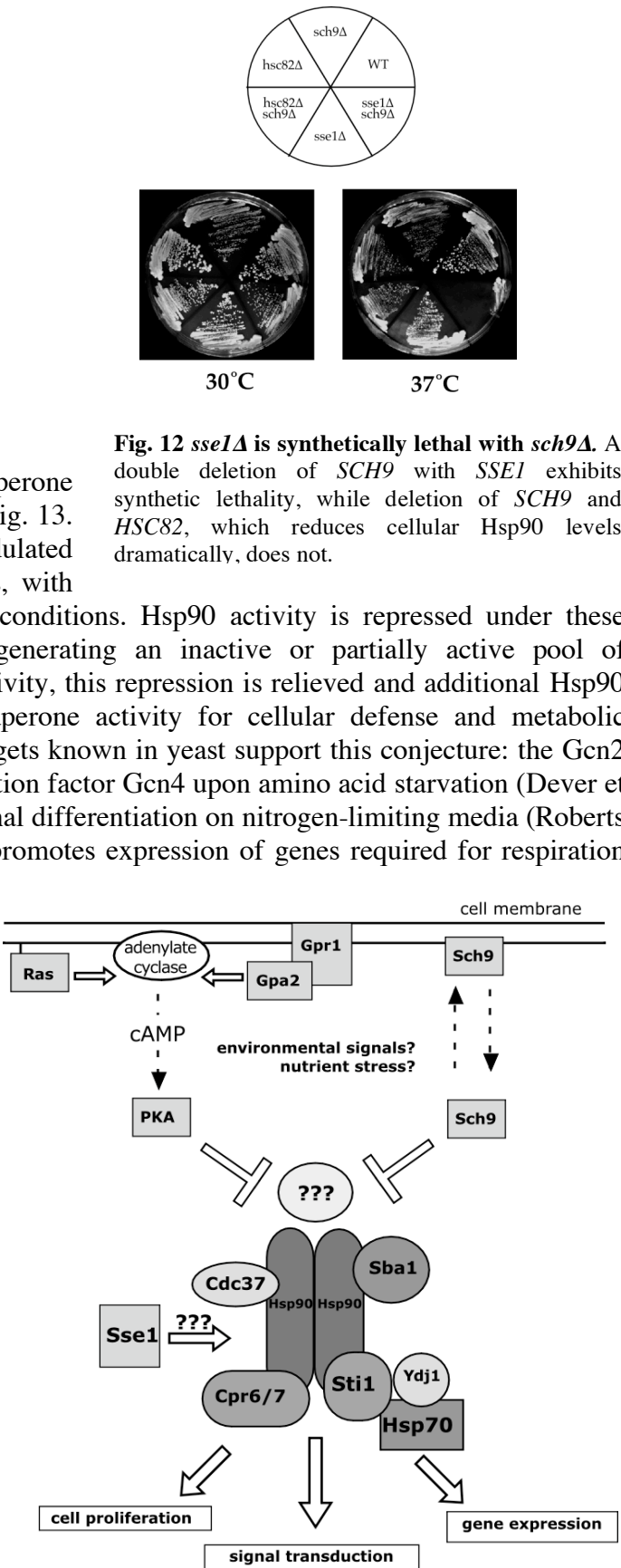
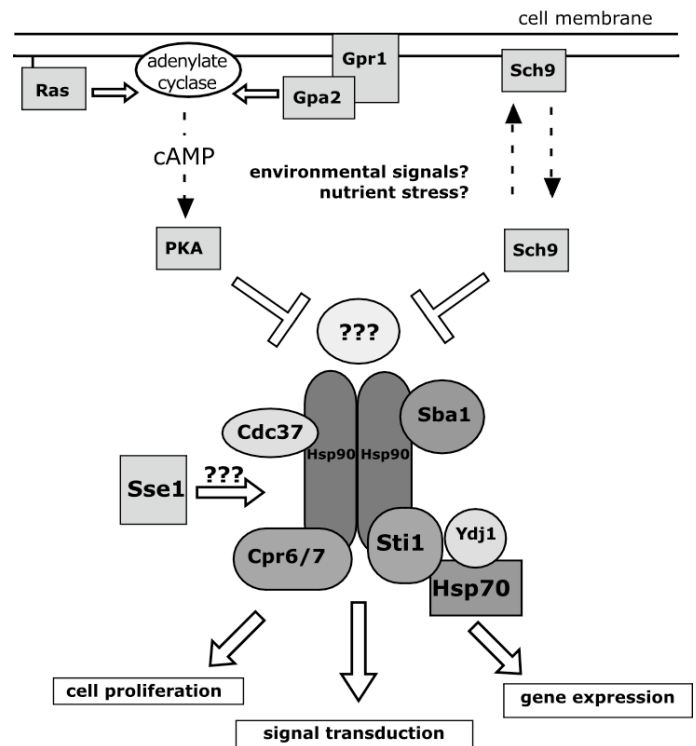


Fig. 12 *sse1Δ* is synthetically lethal with *sch9Δ*. A double deletion of *SCH9* with *SSE1* exhibits synthetic lethality, while deletion of *SCH9* and *HSC82*, which reduces cellular Hsp90 levels dramatically, does not.



B. Progress Report

N/A

C. Aims and Method of Study.

(i) Specific Aims

The Hsp90 molecular chaperone is one of the most abundant proteins in the eukaryotic cell, and its levels must be further elevated to combat diverse environmental stresses. Hsp90, in concert with a number of co-chaperone proteins, is also required for the function of multiple classes of intrinsically labile key regulatory proteins in unstressed cells. These include growth control protein kinases, steroid hormone receptors, transcription factors and other signaling proteins. Therefore, due to its pivotal involvement in cell cycle progression, growth control and signaling, a detailed understanding of Hsp90 function and regulation is of critical importance. This knowledge will be of considerable biomedical relevance, as Hsp90 is required for the activity of both tumor-suppressing (p53) and tumor-promoting (v-Src kinase) molecules.

The protein kinase Sch9, related to the protein kinase A and B (Akt) families of growth control kinases, has been identified as a novel repressor of the Hsp90 chaperone complex in the yeast *Saccharomyces cerevisiae*. **I propose that a regulatory pathway, including the Sch9 protein kinase as an effector, modulates Hsp90 signal transduction activity.** Furthermore, this regulation is a critical link between cell growth and proliferation and stress response mechanisms. **The following specific aims are outlined with the goal of understanding how the Sch9 kinase governs Hsp90-dependent signaling, the pathways that impinge upon this regulation, and the involvement of a recently identified Hsp90 partner, the chaperone Sse1.** The long-term objectives of this project are to understand how the powerful protein remodeling activities of Hsp90 are utilized and integrated into cellular physiology and signaling, and the role of Hsp90 partner chaperones in these processes.

1. Identification of protein(s) mediating Hsp90 regulation by the Sch9 kinase. Genetic and biochemical approaches will be used to identify the specific target of Sch9 required for modulating Hsp90 chaperone function.

2. Functional characterization of Sch9 and analysis of Hsp90 regulation by the cAMP-protein kinase A pathway. The localization and function of the poorly understood Sch9 kinase will be examined. The roles of the related protein kinase A pathway and environmental nutrient sensing in Hsp90 regulation will be determined.

3. Determination of Sse1 chaperone functions in signal transduction through Hsp90. Biochemical analysis of chaperone complexes and chaperone-client protein interactions, and assessment of Hsp90-dependent signaling pathways will be carried out in cells overexpressing or deficient in Sse1. A dosage suppression screen of a *sse1Δ sch9Δ* double mutant strain will be undertaken to further elucidate the connections between the Sse1 chaperone, Hsp90 function and the Sch9 kinase.

(ii) Rationale and/or Significance

As elaborated in the preceding sections, the Hsp90 chaperone system is an integral component of eukaryotic cellular biology, with roles in cell cycle progression/cell proliferation, gene expression and signal transduction. Because of its powerful protein remodeling activity, the Hsp90 chaperone has been recruited to stabilize and in some cases regulate client signal transduction proteins. Importantly, these interactions are entirely conserved from yeast to humans, making the genetically tractable eukaryote *Saccharomyces cerevisiae* an ideal system in which to experimentally address questions of Hsp90 function

and regulation. The proposed work will focus on the regulation of Hsp90 function *in vivo* by the yeast growth control kinase Sch9, a protein bearing significant homology to the higher eukaryotic kinase Akt. Because Akt is critically involved in pathways modulating the decision to proliferate or enter an apoptotic cell death program, it is likely that regulation of the Hsp90 chaperone system is a critical component of Akt responses in multicellular organisms. Elucidation of the mechanism of regulation of Hsp90 by the Sch9 kinase will significantly expand our knowledge of how the cell controls molecular chaperone activity. Furthermore, this proposal seeks to better understand the role of the Sse1 molecular chaperone in Hsp90 function. Sse1 is the yeast homolog of the poorly understood Hsp110 family of HSPs, ubiquitous throughout the eukaryotic lineage. In fact, Hsp110 is one of the few heat shock proteins for which multiple tissue-specific isoforms have been documented in mammalian systems, raising the possibility that Hsp90 complex function is modulated based on tissue or organ location.

(iii) Statement of Cancer Relevance

Although the work outlined in this proposal will be carried out in the model eukaryote, the budding yeast *Saccharomyces cerevisiae*, the results are likely to have a direct impact on cancer biology. Heat shock proteins play a distinct role in cell cycle progression, particularly under stress conditions, due to their ability to stabilize key regulatory proteins such as the cyclin dependent kinases (CDKs), and CDK-regulators such as wee1 (Aligue et al., 1994). A number of HSPs are overexpressed in clinically isolated tumors, and overexpression has been correlated with the ability of tumors to metastasize (Ciocca et al., 1993a; Ciocca et al., 1993b). Overexpression of HSPs such as Hsp70 and Hsp90 is associated with malignancy and failure to respond to combined chemotherapy/hyperthermia treatment programs (Oesterreich et al., 1993; Vargas-Roig et al., 1998). Additionally, it has been shown that overexpression of either Hsp70 or the CDC37 protein, an Hsp90 co-chaperone, is tumorigenic (Stepanova et al., 2000; Volloch and Sherman, 1999). A more direct molecular role for Hsp90 in tumorigenesis is the requirement for the chaperone for function of a number of cellular oncogenes, including v-src^{pp60} and p53 (Blagosklonny et al., 1996; Selkirk et al., 1994; Xu and Lindquist, 1993). Moreover, a class of macrolide antibiotics best represented by the drug geldanamycin functions as a potent antitumor compound due to its ability to bind to and inhibit the activity of Hsp90, thereby completely eliminating cellular transformation due to Hsp90 client oncoproteins such as v-src^{pp60} (McIlwrath et al., 1996). Based on this activity, the drug and more efficacious derivatives are currently in Phase I trials at the National Cancer Institute. Importantly, this drug also inhibits the growth of yeast cells with defects in Hsp90 function, further underscoring the high degree of functional conservation of the Hsp90 system between man and yeast (Dolinski et al., 1998; Liu et al., 1999). Understanding how Hsp90 is regulated in a simple eukaryote such as baker's yeast will facilitate detailed analysis of this and other chaperones' roles in human cancer.

(iv) Plan of Attack

The experiments outlined in this proposal are focused on the central theme of regulation of Hsp90 molecular chaperone activity. Each aim is designed to complement the others, without being dependent upon a specific outcome. Therefore much of the work will be done in parallel, and results obtained from completed sub-projects applied to the remaining goals. The projected time frames for each aim reflect this overlap of effort.

Specific Aim 1: Identification of protein(s) mediating Hsp90 regulation by the Sch9 kinase.

Goals and Rationale

The genetic and molecular evidence presented above strongly supports a role for the Sch9 kinase in modulation of Hsp90 chaperone complex function *in vivo*. My primary goal is to define the mechanism by

which this regulation occurs. I hypothesize that through one or more phosphorylation events, the Sch9 kinase inhibits the function of a protein required for full Hsp90 activity. The identity of this “mediator” is not known, and may be one or more known Hsp90 co-chaperone or a previously uncharacterized component of the Hsp90 complex. The plans described in this specific aim outline a multi-pronged approach focused on identifying this component, followed by preliminary characterization of its role in Hsp90 chaperone function. It is expected that the initial experiments described in this aim will take 1-2 years to complete.

1(a). Genetic and biochemical analysis of known co-chaperones

As a first step toward identifying the relevant Sch9 substrate, the known Hsp90 co-chaperones will be investigated through genetic and biochemical means. The temperature-sensitive growth phenotype of the HSF(1-583) strain is directly proportional to Hsp90 chaperone activity, and therefore provides a convenient primary assay. Most of the Hsp90 co-chaperones listed in Fig. 2 in **Section A** are required for full chaperone activity, and all affect Hsp90-dependent signaling. If Sch9 regulates Hsp90 chaperone activity via inhibitory phosphorylation of one of these known components, then overexpression of the protein should mimic loss of the kinase and suppress the temperature sensitive (*ts*) growth phenotype of HSF(1-583). The following genes encoding Hsp90 chaperone complex components will be tested: *SSA1*, *STI1*, *SSE1*, *CPR6*, *CPR7*, *CDC37*, *SBA1*, *YDJ1* and *CNS1*. Oligonucleotide primers will be designed to amplify the corresponding ORF and purchased from Integrated DNA Technologies (Coralville, IA). For regulated expression of the genes, the copper-inducible series of low- and high-copy vectors recently developed by the Thiele laboratory will be utilized. These vectors contain a minimal promoter region from the copper metallothionein gene *CUP1*, and are potently activated within minutes by the addition of trace amounts of copper to the growth medium (Labbe and Thiele, 1999). Unlike the commonly used galactose-inducible *GAL1-10* promoter, this system does not require the use of alternate carbon sources, which can radically alter cellular physiology. My laboratory currently has antibodies to all of these chaperones generously provided by other investigators, which will be used to verify overexpression. Chaperone overexpression plasmids will be transformed into isogenic HSF wild type and HSF(1-583) strains, and the ability to confer growth to the HSF(1-583) strain at 37°C will be assessed. A chaperone conferring high temperature growth will be considered to be a candidate for regulation by Sch9 and further investigated as described in section 1(d) below.

Because overexpression may alter the stoichiometric balance of the chaperones in the Hsp90 complex, with negative consequences, it is possible that such an approach might fail to uncover the intended target. Therefore, a biochemical analysis of chaperone phosphorylation will be carried out in parallel. A number of efforts to categorize the *S. cerevisiae* proteome by two-dimensional (2D) gel electrophoresis have resulted in an accurate and reproducible map of many soluble and abundant proteins (Futcher et al., 1999; Norbeck and Blomberg, 1997). The Hsp90 co-chaperones are very well represented in these analyses, and many, including Hsp90 itself, Hsp70, and Sti1, exhibit multiple “spots” of varying pI. Whether these signals represent differentially phosphorylated isoforms is unclear. I propose to use 2D electrophoresis to examine the known Hsp90 co-chaperones for evidence of phosphorylation by Sch9. Soluble cell extracts will be generated from isogenic *SCH9* and *sch9Δ* cells, and resolved using a Bio-Rad Mini 2D-gel apparatus, for which funds are requested. The appropriate pI gradient and range can be determined from analysis of the aforementioned literature, but preliminary observations show most of the chaperones migrating with pI values between 4.5 and 6.8, which should not be difficult to resolve. 2D gels will be transferred to nitrocellulose and the specific chaperones detected by immunoblot analysis using specific antibodies. Positive identification of the chaperones will be facilitated by use of the overexpressing constructs generated above, as well as chaperone deletion strains. If Sch9 is responsible for

phosphorylation of one or more Hsp90 co-chaperones, then I expect to see a reduction in spot intensity, or complete elimination of an isoform in the *sch9Δ* strain when compared to the control wild type. Any chaperones displaying such a pattern will be further investigated through in vivo phosphate labeling, followed by immunoprecipitation, as previously demonstrated for Hsp90 in **Section A**. A reduction in band intensity in the *sch9Δ* strain will be taken as indication that Sch9 directly or indirectly phosphorylates that co-chaperone. Because regulation of a co-chaperone may be affected by a single phosphorylation event, it is possible that the modification may be below the limit of detection by both the 2D gel and in vivo labeling methods. The use of the high-copy *CUPI*-driven chaperone expression constructs will therefore facilitate detection by boosting the levels of the substrate. In addition, a second amplification of the ORF with a 5' oligonucleotide encoding the FLAG epitope (DYKDDDDK; Kodak) in frame after the start codon can be carried out to construct plasmids which overexpress a FLAG-tagged version of the chaperone. The FLAG antibody recognizes the epitope with very high affinity, allowing the epitope to be used in single copy, thereby minimizing effects on protein function. Hsp90 co-chaperones displaying evidence of phosphorylation in an Sch9-dependent manner will be further characterized in detail as described in 1(d) below.

1(b). cDNA dosage suppressor screen

In concert with the methods outlined above, I plan to initiate a high-copy suppressor screen of the HSF(1-583) temperature sensitive growth phenotype. While the experiments in section 1(a) are designed to specifically test the known Hsp90 co-chaperones for the ability to suppress the HSF(1-583) phenotype and mimic deletion of *SCH9*, this approach will screen the entire yeast genome for candidates responsible for mediating Sch9 regulation of Hsp90. The initial screening procedure will be the same as that outlined for section 1(a), but will involve transformation of the HSF(1-583) strain with high copy cDNA libraries provided by Dr. Stephen Elledge of the Baylor College of Medicine, a neighbor institution within the Texas Medical Center. A similar screen identified the *HSP82* gene as an HSF(1-583) suppressor using a genomic library, but was not exhaustive (Morano et al., 1999). The Elledge cDNA libraries have the added advantage of being comprised exclusively of expressed genes, and are under the control of strong promoters (Edwards et al., 1997). This is critical in that if the mediator protein is under the transcriptional control of HSF itself, which is a distinct possibility given that many of the known Hsp90 co-chaperones are dependent on HSF for expression under both stress and non-stress conditions, then a genomic library may not express the protein in concentrations high enough to overcome inhibition by Sch9 kinase. This rationale may explain why we only obtained *HSP82* in the first high-copy screen. In addition, it is possible that overexpression of the Sch9 target may not completely overcome negative regulation by the kinase. Therefore, the degree of suppression may be weaker than that observed for overexpression of *HSP82* itself and may not have been picked up in the first analysis. These libraries also contain gene truncations, which have been instrumental in obtaining suppressors when overexpression of the full-length clone was lethal (S. Elledge, personal communication).

1(c). Purification and identification of novel Sch9-associated proteins

The Sch9 kinase appears to negatively regulate Hsp90 chaperone function continuously under optimal growth conditions. This suggests that the kinase may form a stable complex with the substrate responsible for modulating Hsp90 function. Consistent with the data presented in **Section A** demonstrating no effect of *SCH9* deletion on Hsp90 phosphorylation, I have not detected association of the Sch9 kinase with Hsp90 in co-immunoprecipitation experiments (unpublished data). However, in the course of those experiments, I discovered that the Sch9 kinase displays a strong affinity for standard nickel-based metal chelate affinity purification resins. As shown in Fig. 14, this association is abrogated by pretreatment of

the resin with EDTA, consistent with a metal-dependent ligand interaction. I propose to take advantage of this fortuitous binding to identify Sch9 kinase binding partners, with the goal of isolating the protein critical for Hsp90 regulation. Native extracts of cells overexpressing a fully functional triple-hemagglutinin (HA)-tagged Sch9 kinase will be bound to commercial metal-chelate affinity resin and washed with buffers empirically determined to maximize specific retention of Sch9. Following elution, the samples will be resolved by SDS-PAGE and Sch9-associated proteins identified by silver stain. The procedure will be performed in parallel with *sch9Δ* cell extracts as a negative control. Proteins co-purifying with Sch9, but absent in the *sch9Δ* lanes will be considered putative Sch9-interacting partners. This approach has the advantage of being scalable to larger extract volumes, which will be necessary to detect weakly-binding substrates. If we fail to detect Sch9-binding proteins by this method, the same procedure will be carried out on cell extracts from methionine- S^{35} -radiolabeled cells, followed by fluorography to increase the sensitivity of the approach. Once candidate proteins are identified, the co-purification will be optimized and scaled up to obtain enough protein for mass spectrometric identification at the Keck Foundation Biotechnology Resource Laboratory at Yale University, which on a fee-for-service basis will perform tryptic digestion, LCQ-MS, and a GenBank peptide mass search on as little as 1 pmol of submitted protein (<http://info.Med.Yale.Edu/wmkeck/>). This procedure is more sensitive than peptide sequencing, and is greatly facilitated by the availability of the completely sequenced yeast genome.

If the purification procedure performed on batch samples or spin cartridges fails to sufficiently enrich Sch9, I have arranged to use an Pharmacia AKTA Purifier high-performance protein purification system in the laboratory of Dr. C.S. Raman, an assistant professor in the Biochemistry and Molecular Biology Department here at the UT Medical School. Dr. Raman is an expert in structural biology and protein purification and has extensive experience with metal-chelate chromatography. With this system and Dr. Raman's guidance, I can design a more sophisticated purification regimen including downstream columns with gel filtration or ion exchange options. As a third potential approach, I can also utilize the triple-HA epitope tag for large-scale native immunoprecipitation of Sch9 and associated proteins. Protein kinases are routinely detected in complexes with their substrates when purified or enriched from native cell extracts, supporting this line of investigation.

In the event that the biochemical approach fails to identify any bona fide Sch9-interacting proteins, we may also employ the yeast two-hybrid system as an alternative method. In this case, the Sch9 kinase will be used as "bait" and an appropriate fusion library screened for interacting proteins. In anticipation of this possibility, we have obtained an activation-domain fusion library from Stephen Elledge which can be used with multiple two-hybrid systems. The two-hybrid approach has been successfully used to identify kinase substrates. For example, the pheromone/filamentation response regulators Dig1 and Dig2 were cloned by virtue of their interaction with the Kss1 MAP kinase, and were subsequently shown to be directly phosphorylated by Kss1 (Cook et al., 1996). Analysis of the Yeast Protein Interaction project web page maintained by Stan Fields and CuraGen reveals no known interactions for Sch9 at this time, but the site will be checked regularly for updates (<http://portal.Curagen.Com>).

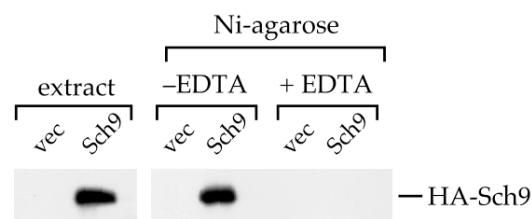


Fig. 14 HA-Sch9 binds to metal-chelate affinity resin. Native protein extracts were made from wild type cells transformed with a plasmid expressing HA-SCH9 or vector alone as control. The extract was split into thirds, and either boiled in sample buffer (extract), or bound to untreated (-EDTA) or EDTA-stripped Ni^{2+} -NTA agarose beads (EDTA), washed, and eluted in sample buffer. Equivalent amounts were resolved by SDS-PAGE and immunoblotted with anti-HA antibody.

1(d). Preliminary characterization of Sch9 mediator protein

The combination of diverse approaches outlined in the sections above should serve to identify one or more proteins directly modified by the Sch9 kinase that modulate Hsp90 chaperone activity in vivo. While a full characterization of this protein and its roles in linking Sch9 and Hsp90 is beyond the scope of this proposal, I plan to initiate these studies by confirming direct interaction and phosphorylation of the mediator by Sch9 and analyzing the role of the mediator in Hsp90 complex function.

As hypothesized above, the mediator protein may be one of the known Hsp90 co-chaperones or a protein previously not known to be involved in Hsp90 function. The specific steps to be taken will depend in part on this important distinction, as some molecular and mechanistic detail is available for the known co-chaperones, while a novel Hsp90-binding protein will require a great deal more initial characterization. Regardless, one of the first important steps will be to verify phosphorylation of the mediator by Sch9 in vivo. The mediator gene will be PCR-amplified and the protein tagged with an amino-terminal FLAG epitope as described in section 1(a), and in vivo phosphorylation tested by immunoprecipitation from P³²-phosphate-labeled *SCH9* and *sch9Δ* cells. If a significant level of phosphorylation is observed, retardation in migration correlating with degree of phosphorylation and *SCH9* allele status may be observed via SDS-PAGE analysis followed by anti-FLAG immunoblot. To determine if the association is direct, co-immunoprecipitation experiments will be carried out using HA-Sch9 and the FLAG-tagged mediator protein. Future experiments will include assessment of the precise residues modified by Sch9, as well as development of an in vitro phosphorylation assay. Knowledge of the site of phosphorylation will allow site-specific mutagenesis of the modified residue, followed by characterization of the effects of phosphate modification on mediator function.

A number of predictions must be satisfied to provide convincing evidence that this mediator is responsible for Sch9 effects on Hsp90 chaperone function. First, overexpression of Sch9 strongly inhibits growth of the HSF(1-583) strain through increased inhibition of Hsp90 function. This phenotype should be reduced, if not completely abrogated by deletion of the mediator protein. Second, overexpression of Sch9 also inhibits Hsp90-dependent signaling (unpublished data). This effect should be minimized in the absence of the mediator protein. Third, overexpression of the mediator itself should mimic the HSF(1-583) *ts* suppression phenotype caused by loss of the Sch9 kinase, a condition which may already be satisfied if the mediator is isolated by overexpression screening. These predictions will be tested using reagents and strains already in hand, in addition to the mediator knockout strain, which will be constructed using standard yeast techniques. The experiments outlined in this section are by necessity exploratory. As established earlier, substantial genetic and molecular evidence supports a role for the Sch9 kinase in modulation of Hsp90 activity. Once the component(s) linking Sch9 with the Hsp90 complex is identified, we are well-poised to define the mechanism of regulation in detail. For example, the effects of phosphorylation of the mediator on association with the Hsp90 chaperone complex, on assembly of the co-chaperones with Hsp90, and of Hsp90-substrate interactions will all be assessed using reagents already in hand.

Specific Aim 2: Functional characterization of Sch9 and analysis of Hsp90 regulation by the cAMP-protein kinase A pathway.***Goals and Rationale***

The *SCH9* gene was first isolated over ten years ago as a multicopy suppressor of a *cdc25^{ts}* mutation, which inactivates the Ras-cAMP pathway. Subsequent reports established that Sch9 operates independently of the Ras-cAMP system, is not required for PKA activation, and does not play a major role

in regulation of PKA-dependent events such as haploid invasive growth (Lorenz et al., 2000). A recent report suggested that Sch9 may be responsible for signaling through the Gpa2/Gpr1 G-protein coupled receptor system (Xue et al., 1998). However, multiple reports have since demonstrated that Gpa2 instead directly activates adenylate cyclase in response to nutrient status, thereby controlling the camp-PKA pathway (Colombo et al., 1998; Kraakman et al., 1999). The identification of *SCH9* as a loss of function suppressor of the HSF(1-583) *ts* growth defect is the first major role found for this elusive kinase. However, because overexpression of an activated Ras allele mimics the growth inhibition phenotype of Sch9 overexpression in the HSF(1-583) strain, it is likely that the Ras-cAMP pathway may also play a role in Hsp90 regulation through protein kinase A. In keeping with the mystery surrounding the cellular roles of Sch9, very little is known about the kinase itself. Where is it localized in the cell? What regulates its activity in vivo? What role does the amino-terminal C2 domain play in kinase function, since this motif is known to mediate phospholipid/membrane interactions in other proteins? The experiments described below are designed to answer these questions, and explore the cooperative roles that Sch9 and the Ras-cAMP-PKA pathway play in Hsp90 regulation. The initial experiments outlined in section 2(a) are projected to require one year to complete, with an additional year to clone and characterize the slow growth suppressor. Section 2(b) is predicted to require another year of effort.

2(a). Characterization of the Sch9 kinase

The first priority in the characterization of the Sch9 kinase will be to localize it within the cell. Both the catalytic (Tpk) and regulatory (Bcy1) subunits of protein kinase A are differentially located in the nucleus or cytoplasm in response to glucose status and cAMP levels in yeast (Griffioen et al., 2000), and recruitment to the plasma membrane is an early step in activation of the mammalian homolog of Sch9, Akt (Kohn et al., 1996). Moreover, components of the Hsp90 complex have been found in both the nucleus and the cytoplasm (Sanchez et al., 1990). Initial localization experiments will be performed using the previously generated HA-tagged allele of *SCH9*, which fully complements the *sch9Δ* slow-growth phenotype. Wild type cells expressing HA-Sch9 and grown in glucose-replete conditions will be used for indirect immunofluorescence analysis with commercial anti-HA and fluorescein-conjugated antibodies. I have experience in fluorescence microscopy (Morano et al., 1999), and have unrestricted access to an Olympus BX-60 fluorescent microscope maintained by my departmental colleague Dr. William Margolin, an expert in fluorescent localization of proteins in bacterial cells. This microscope is equipped with standard fluorescence filter sets, Nomarski/DIC optics, and a high-sensitivity cooled CCD camera for image acquisition.

Because of the close functional relationships between PKA and Sch9, I expect to see the majority of the protein localized within the cytosol, as evidenced by diffuse staining throughout the cell interior, but will investigate the possibility of sub-populations of HA-Sch9 localizing within the nucleus or concentrated at the cell periphery. The former case can be established via co-localization with the DNA stain, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and the latter by co-localization with a GFP-tagged copper transporter, Ctr3, which is easily visible as a discrete ring around the cell membrane (Pena et al., 2000). Dr. Dennis Thiele has generously provided a plasmid expressing Ctr3-GFP. In support of this potential outcome, I have obtained preliminary evidence through subcellular fractionation studies that a small percentage of HA-Sch9 is membrane associated under normal conditions (data not shown). In addition, data records from the Yale Genome Analysis Center, which is analyzing in-frame transposon tagged alleles of all yeast genes, report two independently generated C-terminal Sch9-GFP fusions displaying "granular" localization (<http://ygac.Med.Yale.Edu/triples>). If multiple compartments are indeed populated by HA-Sch9, and if the cytosolic signal overwhelms these fainter signals, then confocal microscopy may be required. A confocal microscope is available on a contract-use basis in the

Biochemistry and Molecular Biology Department here at the UT Medical School. To determine if Sch9 localization responds to nutrient status in a manner similar to that established for PKA, cells expressing HA-Sch9 will be shifted from high-glucose medium to low- or no-glucose-containing medium. Samples will be harvested at one-minute intervals and immediately fixed by the addition of formaldehyde, followed by indirect immunofluorescence analysis. A major limitation of this approach is that the kinetics of translocation to and from the nucleus or the cell membrane may be faster than the formaldehyde fixation rate. To address this, a GFP-tagged allele of Sch9 will be constructed and the effects of changes in nutrient status on localization immediately analyzed in live cells.

The C2 domain of Sch9 is located within the amino-terminal half of the Sch9 kinase (residues 183 to 396), and is conserved in the two *S. pombe* homologs Sck1 and Sck2 (see Fig. 3; Fujita and Yamamoto, 1998; Jin et al., 1995). All three kinases display high homology in their catalytic domains to the fungal protein kinase A family, but are unique in possessing an approximately 400 residue amino-terminal extension distinct from the catalytic domain. C2 domains are found in a variety of eukaryotic signaling proteins, including the protein kinase C family in higher eukaryotes as well as yeast Pkc1 (Levin et al., 1990), phospholipases, and also in yeast the vacuolar protein sorting kinase Vps34, the membrane-localized ubiquitin ligase Rsp5 and the polarity protein Bud2 (Rizo and Sudhof, 1998). The primary roles of the domain in mammalian proteins appear to be membrane localization and/or calcium regulation, but C2 domains may also be involved in phospholipid-mediated signaling events (Newton, 1995). Essentially nothing is known about the role of this domain in yeast proteins.

To address the role of the C2 domain in Sch9 function, PCR-mediated mutagenesis will be used to delete the entire C2 region. This allele will be expressed from a low-copy plasmid and analyzed in *sch9Δ* cells with both wild type Sch9 and empty vectors as controls. Because *sch9Δ* cells exhibit a prominent slow-growth phenotype, differences in growth rate between Sch9 and Sch9 C2Δ strains will be examined to assess the contribution of the C2 domain to Sch9 function. In addition, the ability of the Sch9 C2Δ allele to reverse suppression of the *ts* phenotype of HSF(1-583) cells by the *sch9Δ* mutation will be determined. Should the C2 domain be observed to affect function of Sch9, more precise point mutations known to inactivate this domain will be created to verify that it is the C2 function, specifically, that is required (Medkova and Cho, 1998). The C2 deletion will be constructed in the context of the HA-tagged allele of Sch9, and the localization of C2 deletion or point mutants will be examined by indirect immunofluorescence. If the C2 domain is required for Sch9 function, and if we can correlate the presence of the C2 region with subcellular localization, then we will have made the first steps in defining a potential activation pathway for the Sch9 kinase. Although it is certainly possible that the C2 domain is not involved in Sch9 function or regulation, it would be surprising then that it is retained in both *S. pombe* homologs, which are also thought to operate in parallel with the PKA pathway in that organism.

To further elaborate the Sch9 regulatory pathway, a spontaneously isolated suppressor allele of the *sch9Δ* slow growth phenotype will be cloned and characterized. This suppressor arose in the HSF(1-583) *sch9Δ* strain background, and as shown in Fig. 15, completely reverses suppression of the HSF(1-583) *ts* phenotype. Moreover, when heme signaling through the Hsp90-dependent Hap1 transcription factor was analyzed via β -galactosidase reporter assay (Zhang et al., 1998), restoration of activity by the *sch9Δ*

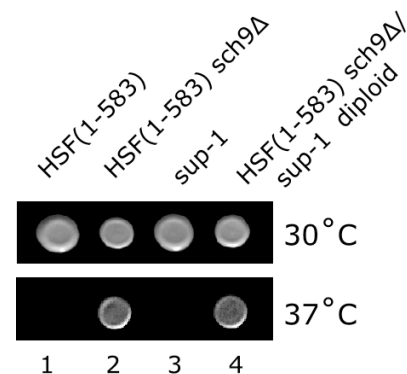


Fig. 15 Isolation of a suppressor of *sch9Δ* slow growth and HSF(1-583) derepression. The indicated strains were spotted onto minimal medium and grown for 3 days (30°C) or 4 days (37°C). Column 4 demonstrates the recessive nature of both suppressor phenotypes upon backcross.

mutation was also reversed (unpublished data). Preliminary genetic characterization of this mutant revealed standard 2:2 segregation of the suppressor phenotype upon backcross, and loss of suppression in diploid, indicative of a recessive mutation in a single locus (unpublished data). Because complementation of the recessive mutation will result in the ability of the suppressor strain to grow at 37°C, cloning should be easily accomplished via transformation with a genomic library and selection for high temperature growth. Since this suppressor complements both the slow-growth and Hsp90 derepression phenotypes of *sch9Δ* cells, it is likely to be a downstream effector of Sch9 signaling, making it a very attractive candidate for analysis.

2(b). Roles of the cAMP-PKA pathway and nutrient sensing in Hsp90 regulation

As presented in **Section A**, I have obtained data implicating the cAMP cascade and the protein kinase A system in Hsp90 regulation. Sch9 and the cAMP-PKA pathway are believed to operate independently in regulating cellular proliferation rate, but may also share common targets. For example, the Msn2 and Msn4 transcription factors, which govern the general stress response, are a major regulatory target of the cAMP-PKA system (Gorner et al., 1998). Loss of PKA activity constitutively derepresses the general stress program, resulting in a phenomenon termed heat shock resistance, the ability to withstand acute, transient exposure to very high temperatures of 50-60°C (Cameron et al., 1988). It should be emphasized that this phenotype is not related to the long-term, temperature sensitive growth phenotype exhibited by the HSF(1-583) mutation. Moreover, Msn2 and Msn4 are not required for suppression of HSF(1-583) by the *sch9Δ* mutation, further distinguishing regulation of the general stress response by PKA from Hsp90 regulation via Sch9 (Morano and Thiele, 1999b). However, loss of the Sch9 kinase results in mild derepression of the general stress response pathway (Crauwels et al., 1997), and my data demonstrate that loss of cAMP-PKA activity partially suppresses HSF(1-583) (**Section A**). Together, these findings suggest that Sch9 and PKA may play additive roles in Hsp90 regulation much as they do in growth control.

To further elaborate these roles, I plan to examine the two known PKA activation pathways for effects on Hsp90 regulation. Recent reports have begun to delineate two independent PKA activation systems, the first operating through the Cdc25 and Ras2 proteins to activate adenylate cyclase in response to growth under glucose-rich conditions, and the second involving the Gpr1-Gpa2 G-protein coupled receptor system, which appears to be responsible for activation of adenylate cyclase specifically upon *transition* to glucose medium (Kraakman et al., 1999). Because suppression of the HSF(1-583) mutation occurs during prolonged growth on glucose-replete medium, I predict that mutations in the Ras pathway will have a more pronounced effect on Hsp90 regulation than mutations in the Gpr1/Gpa2 pathway. To test this hypothesis, I plan to separately construct null mutations in the *RAS2*, *GPA2* and *GPR1* loci in combination with the existing HSF(1-583) mutation, using PCR-generated kanamycin disruption cassettes. All three of these mutations are known to be viable, facilitating strain construction. The *ras2Δ* HSF(1-583), *gpa2Δ* HSF(1-583) and *gpr1Δ* HSF(1-583) strains will be analyzed for growth at 37°C as the primary indicator of Hsp90 regulation, and compared with HSF(1-583) and *sch9Δ* HSF(1-583) strains as controls. Strains will also be analyzed for Hsp90-dependent signaling through the Hap1 transcription factor by the previously described β-galactosidase assay of a Hap1-responsive UAS-*lacZ* reporter. If either pathway is involved in Hsp90 inhibition, I expect to observe some degree of growth at 37°C, and some increase in activity from the Hap1 reporter at 30°C upon loss of the gene relative to wild type. Because Gpr1 and Gpa2 operate in a linear pathway (Xue et al., 1998; Yun et al., 1998), I expect both of these mutations to confer the same phenotype, whether it be suppression or no effect. It is possible that residual adenylate cyclase activation may occur when only one or the other pathway is disrupted, as evidenced by data demonstrating an additive growth defect when *ras2Δ* mutations are combined with *gpa2Δ* or *gpr1Δ* deletions. I therefore plan to construct a triply mutated strain bearing *ras2Δ gpa2Δ* and the HSF(1-583)

alleles. This strain should be severely deficient in cAMP production, and therefore deficient in PKA activity as well, and will be tested as described above. To verify that the effects of these pathways are solely due to low PKA activity brought about by diminished cAMP production, the *PDE2* gene, encoding the critical yeast high-affinity phosphodiesterase, will be disrupted. *pde2Δ* strains hyperaccumulate cAMP, which should restore cAMP levels to *gpa2Δ* or *ras2Δ* strains (Sass et al., 1986). Lastly, triple mutant strains will be constructed with the *sch9Δ* HSF(1-583) genotype as well as *ras2Δ* or *gpa2Δ* mutations to determine if additive derepression of Hsp90 activity occurs when the Sch9 and PKA pathways are disrupted in tandem. In support of this line of investigation, genetic analyses have demonstrated synergistic growth phenotypes upon combination of *sch9Δ* and *ras2Δ* or *gpa2Δ* alleles (Kraakman et al., 1999; Lorenz et al., 2000).

Protein kinase A activity is precisely modulated in the cell in response to glucose availability, and it is thought that Sch9 may be involved in nutrient sensing as well, although the data are less clear (Xue et al., 1998). If these kinases are participating in Hsp90 chaperone complex inhibition in response to cellular nutrient status, then it should be possible to mimic suppression of the HSF(1-583) *ts* phenotype by nutrient depletion. I have presented preliminary data (**Section A**) that incubation of HSF(1-583) cells on galactose as the sole carbon source leads to partial derepression of Hsp90 function as evidenced by growth at 37°C. Importantly, we have not detected increased transcription of genes encoding Hsp90 complex components in response to glucose starvation, nor are they specifically induced by galactose (data not shown). To further extend these observations, I plan to assess the ability of alternate, non-fermentable carbon sources, such as glycerol-lactate and ethanol, to suppress the HSF(1-583) phenotype. If suppression is observed, then more detailed analysis of Hsp90 signaling through the Hap1 transcription factor and signaling and stability of the glucocorticoid receptor will be carried out under these media conditions. Once we have identified the mediator protein responsible for Sch9/PKA inhibition of Hsp90 signaling, a more sophisticated analysis will be undertaken to monitor phosphorylation status of the protein in response to carbon source. These studies will be expanded by performing time course analyses of mediator phosphorylation in vivo after shift from non-suppressing to suppressing conditions. Recent reports have implicated both Sch9 and Gpr1/Gpa2 in a so-called “fermentable growth medium” pathway, defined as the absence of a critical nutrient such as nitrogen, in the presence of sufficient glucose (Crauwels et al., 1997). Because Sch9 activity may be modulated by nitrogen status, similar time course analyses will be performed on HSF(1-583) cells grown in nitrogen-deficient medium and then pulsed with ammonium sulfate, and/or amino acids. Together, these experiments will develop a broader picture of Hsp90 chaperone regulation by these growth control kinases, and begin to define the regulatory network that couples growth conditions, cellular proliferation, and chaperone activity.

Specific Aim 3: Determination of Sse1 chaperone functions in signal transduction through Hsp90.

Goals and Rationale

The Hsp110 family of molecular chaperones are a highly conserved but poorly understood group of heat shock proteins. Hsp110 displays the closest homology at the amino acid level with the diverse Hsp70 family of heat shock proteins (Lee-Yoon et al., 1995). However, the Hsp110s are unable to carry out protein refolding, one of the defining characteristics of the Hsp70s. Instead, in vitro experiments have demonstrated that both the mammalian Hsp110 protein and the yeast ortholog Sse1 bind unfolded polypeptides and maintain them in a folding-competent state (Brodsky et al., 1999; Oh et al., 1997). This behavior is identical to that exhibited by Hsp90 and its associated co-chaperones. In keeping with the functional similarities between Hsp90 and Sse1, the two chaperones genetically and functionally interact

(Liu et al., 1999). I have further observed a pronounced synthetic phenotype between *sse1Δ* and *sch9Δ* mutations specifically at 37°C, suggesting a synergy between Sse1 and Sch9 in regulation of Hsp90 (see **Section A**). This finding is further supported by the observation that overexpression of Sse1 inhibits the growth of HSF(1-583) cells (see **Section A**). What is the role of Sse1 in Hsp90 chaperone function? What are the connections between this chaperone and Sch9? Intriguingly, both Sse1 and its *S. pombe* homolog Pss1 show genetic interactions with the ras-cAMP pathway, making it likely that the experiments in *S. cerevisiae* will be relevant to other eukaryotes (Chung et al., 1998; Shirayama et al., 1993). Moreover, human Hsp110 is currently under investigation for use as an adjuvant in ant-tumor vaccines (Wang et al., 2001) The experiments described below are designed to elucidate the role of Sse1 in Hsp90 chaperone complex activity and to test the hypothesis that Sch9 may function specifically with the Sse1 chaperone cooperatively in Hsp90 regulation. Sections 3(a) and 3(b) should be completed within one year, with an additional six months to isolate and begin characterizing genetic suppressors of the *sse1Δsch9Δ* temperature sensitive phenotype.

3(a). Determination of the roles of Sse1 in client maturation and function

The stoichiometric balance of Hsp90 and many of its associated co-chaperones is of critical importance for proper substrate maturation and function. This can be seen at the genetic level when individual chaperones are deleted or overexpressed, resulting in phenotypes ranging from slow growth to temperature sensitivity to complete growth inhibition. Overexpression or deletion of the *SSE1* gene results in profound growth inhibition of HSF(1-583) cells, which are compromised for Hsp90 function (**Section A**). I plan to examine this phenotype in more detail at the molecular level via analysis of Hsp90 chaperone complex-substrate interactions in cells overexpressing *SSE1*. My hypothesis is that Sse1 is coordinating with the other chaperones in substrate maturation and that excess or insufficient Sse1 protein results in abortive complexes. To address this proposal, we will use the sensitive FLAG-tagged GR and GR-dependent lacZ reporter system. The analysis will initially be carried out in the HSF(1-583) strain, which exhibits minimal but measurable levels of hormone-dependent lacZ activity. These cells will be further transformed with control or *SSE1* overexpression vectors, and hormone-dependent GR activity determined via β-galactosidase assay. Based on the substantial growth inhibition of HSF(1-583) cells due to *SSE1* overexpression, I expect to observe a significant decrease in GR activity in the same strain. Because of the slow growth caused by Sse1 overexpression, a conditional expression system with the *SSE1* coding sequence under the transcriptional control of the inducible *CUP1* promoter (described in section 1(a)) will be used. With this system, abundant transcription of *SSE1* can be induced by addition of copper immediately prior to hormone induction, diminishing complications arising from chronic overexpression. If reduction in GR activity is observed, GR stability will be measured via half-life extrapolation from pulse-chase radiolabeling experiments, as described in **Section A**. These studies will then be carried out in HSF wild type cells to verify that the effects are not solely due to the compromised Hsp90 complex activity observed in the HSF(1-583) strain. It should be pointed out that wild type cells display an apparent excess of Hsp90 chaperone “capacity”, with regard to the cellular requirements. Hsp90 and most of the core co-chaperones are highly expressed proteins, and it has been demonstrated that while complete elimination of both Hsp90-encoding genes is lethal, Hsp90 levels can be reduced by 95% without a visible growth phenotype, even under stress conditions (Borkovich et al., 1989; Picard et al., 1990). The HSF(1-583) strain provides an attenuated level of Hsp90 activity, effectively “sensitizing” the cell for our experiments.

3(b). Biochemical analysis of Hsp90 chaperone complexes when overexpressing or deleting Sse1

To further define the biochemical interactions of Sse1 with the Hsp90 complex, I will perform co-immunoprecipitation experiments to characterize substrate-Hsp90 complexes and Hsp90-co-chaperone

complexes in cells with adjusted Sse1 levels. The FLAG-GR construct will be expressed in wild type, *sse1Δ* or *SSE1* overexpressing cells, and immunoprecipitated from native extracts. These immune complexes will be resolved by SDS-PAGE and the presence of Hsp90 and the known co-chaperones detected via Western blot analysis. As described in section 1(a), the laboratory has antibodies to all the major chaperones, greatly facilitating the investigation. We expect to observe differences in the amounts of the various chaperones associating with GR when Sse1 levels are severely modulated. The laboratory of Susan Lindquist, University of Chicago, has used this procedure successfully to characterize the role of Cdc37 (Kimura et al., 1997) and Sti1 (Chang et al., 1997) in Hsp90 complex function. Identification of co-chaperones specifically enriched or depleted in GR complexes in the absence or surplus of Sse1 will significantly enhance our ability to assign a functional role to this chaperone.

It is possible that we may not observe major defects in GR function when Sse1 stoichiometry is imbalanced, or that the efficiency of FLAG-GR co-immunoprecipitation is not sensitive enough to detect subtle changes in Hsp90 complex composition. As an alternative approach, a poly-histidine tagged Hsp90 construct successfully used to first establish association of Sse1 with Hsp90 (Liu et al., 1999) will be used to isolate Hsp90-co-chaperone complexes via nickel-chelate affinity purification. This approach is in fact isolating a heterogeneous mix of Hsp90 complexes consisting of the various *in vivo* clients and associated co-chaperones in different sub-complexes based on stages in the folding cycle. Analysis of co-chaperones associating with Hsp90 in wild type, *sse1Δ*, and *SSE1*-overexpressing cells will further define the specific interactions between Sse1 and the Hsp90 complex.

3(c). High-copy suppression of synthetic *sse1Δsch9Δ* mutant

Because the *in vivo* roles of the Hsp110 family are unknown, it would be highly desirable to undertake a genetic suppression screen of the *sse1Δ* strain to identify potential targets or partners of the chaperone. However, the temperature sensitivity of *sse1Δ* mutants is not severe enough to permit conventional selection strategies. The observed conditional lethality occurring upon deletion of both *SSE1* and *SCH9*, presented in **Section A**, is strongly suggestive of a synergistic interaction. Alone, the *sse1Δ* mutation is known to result in a mild temperature sensitive phenotype (Mukai et al., 1993), and *sch9Δ* cells are slow-growing at all temperatures, but *sse1Δ sch9Δ* cells fail to form colonies after prolonged incubation at 37°C, making the strain ideal for genetic suppressor isolation. We plan to take advantage of this phenotype to isolate high-copy suppressors conferring growth at 37°C, using the cDNA libraries provided by Stephen Elledge and previously described in section 1(a). We expect these suppressors to fall into one of three classes: i) suppressors of the *sch9Δ* mutation, ii) suppressors of the *sse1Δ* phenotype, and iii) suppressors of the synergistic double mutant phenotype. Because a class (i) suppressor may already be represented in the *sch9Δ* suppressor allele being characterized in section 2(a), we plan to focus specifically on the latter two classes. These suppressor plasmids might be expected to confer growth at 37°C, but not to restore wild type growth rates when transformed into *sch9Δ* single-mutant cells. Suppressor plasmids will be isolated and characterized via sequencing and identification through the Saccharomyces Genome Database (<http://genome-www.Stanford.Edu/saccharomyces/>). What should we expect to obtain from this screen? If Sse1 is required for the stability, folding, or function of a specific target that is also required for growth at 37°C, then overexpression of this target may alleviate the conditional *ts* phenotype. Alternatively, if the Sse1 chaperone functions primarily as a component of the Hsp90 chaperone complex, we may identify key components of the complex that mediate this interaction. The Sse1 family of chaperones is highly conserved in all eukaryotes, and this approach represents a unique opportunity to begin to determine the cellular roles they play using the genetic power of yeast.

FACILITIES

Laboratory: Approx. 750 sq. ft. within the Dept. of Microbiology and Molecular Genetics, renovated in 1990 and again in 2000. Nearby facilities include walk-in 37C and 4C rooms, an equipped dark room, automatic film processor, and an autoclaving/dishwashing facility. The departmental DNA Core Facility includes two ABI DNA Sequencers and an oligonucleotide synthesizer. The departmental Imaging Center includes a Packard Instant Imager and an Olympus BX60 fluorescent microscope equipped with differential interference contrast (Nomarski) optics and low-light CCD imaging capability. High-capacity fume hood for chemical containment.

Clinical: N/A

Animal: N/A

Computer: One Apple Macintosh G4 Cube computer located in office, and a high-capacity Hewlett Packard LaserJet 4050N printer. The laboratory contains one Apple Macintosh iMac, a Hewlett Packard color printer, and an HP 24-bit color scanner. All computers are connected to the departmental Sun Microsystems SPARC Server and have Internet and MEDLINE database access. Departmental software includes the Wisconsin Sequence Analysis Package and a variety of molecular biology software packages. Additional computers are available to students in the Departmental Computing Center, containing three Apple Macintosh and two Dell Dimension computers.

Office: 125 sq. ft., renovated in 1991 and again in 2000. Support staff includes a shared secretary (responsible for three faculty) and dedicated ordering and accounting personnel.

Other: The department maintains an on-site dedicated automated sequencing facility, and a protein analysis facility featuring a BiaCore 2000 instrument for analyzing protein-protein interactions. The University of Texas Health Sciences Center at Houston is located within the Texas Medical Center, one of the largest biomedical and clinical enterprises in the world. Neighboring institutions include Rice University, the Baylor College of Medicine, and the M.D. Anderson Cancer Center.

MAJOR EQUIPMENT

Microbiological culture: two circulating-air incubators for yeast and bacteria growth, two New Brunswick shaking waterbaths, one large New Brunswick floor shaking incubator for yeast culture.

Equipment: a refrigerator, a -20C freezer, a -80C freezer, a two-door refrigerated chromatography cabinet, three micro-centrifuges, and protein/nucleic acid electrophoresis equipment, hybridization oven, electroporator, Beckman DU640 UV/VIS spectrophotometer.

Shared equipment: A French press, scintillation counter, densitometer, sonicator, and ultracentrifuges are available in the adjacent laboratories of Drs. T. Koehler and A. Dombrowski (15 researchers).

REFERENCES

- Aligue, R., Akhavan-Niak, H. and Russell, P. (1994) A role for Hsp90 in cell cycle control: Wee1 tyrosine kinase activity requires interaction with Hsp90. *EMBO J*, **13**, 6099-6106.
- Bellacosa, A., de Feo, D., Godwin, A.K., Bell, D.W., Cheng, J.Q., Altomare, D.A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V. and et al. (1995) Molecular alterations of the Akt2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer*, **64**, 280-285.
- Bellacosa, A., Testa, J.R., Staal, S.P. and Tsichlis, P.N. (1991) A retroviral oncogene, Akt, encoding a serine-threonine kinase containing an SH2-like region. *Science*, **254**, 274-277.
- Blagosklonny, M.V., Toretsky, J., Bohen, S. and Neckers, L. (1996) Mutant conformation of p53 translated in vitro or in vivo requires functional hsp90. *Proc. Natl. Acad. Sci. USA*, **93**, 8379-8383.
- Borkovich, K.A., Farrelly, F.W., Finkelstein, D.B., Taulien, J. and Lindquist, S. (1989) Hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Mol. Cell. Biol.*, **9**, 3919-3930.
- Broach, J.R. and Deschenes, R.J. (1990) The function of ras genes in *Saccharomyces cerevisiae*. *Adv. Cancer Res.*, **54**, 79-139.
- Brodsky, J.L., Werner, E.D., Dubas, M.E., Goekeler, J.L., Kruse, K.B. and McCracken, A.A. (1999) The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J. Biol. Chem.*, **274**, 3453-3460.
- Buchner, J. (1996) Supervising the fold: Functional principles of molecular chaperones. *FASEB J.*, **10**, 10-19.
- Cameron, S., Levin, L., Zoller, M. and Wigler, M. (1988) cAMP-independent control of sporulation, glycogen metabolism, and heat shock resistance in *S. cerevisiae*. *Cell*, **53**, 555-566.
- Caplan, A.J. (1999) Hsp90's secrets unfold: New insights from structural and functional studies. *Trends Cell Biol.*, **9**, 262-268.
- Chang, H.C. and Lindquist, S. (1994) Conservation of Hsp90 macromolecular complexes in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **269**, 24983-24988.
- Chang, H.C., Nathan, D.F. and Lindquist, S. (1997) In vivo analysis of the Hsp90 cochaperone Sti1 (p60). *Mol. Cell. Biol.*, **17**, 318-325.
- Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi, T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N. and Testa, J.R. (1992) Akt2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc. Natl. Acad. Sci. U S A*, **89**, 9267-9271.
- Chung, K.S., Hoe, K.L., Kim, K.W. and Yoo, H.S. (1998) Isolation of a novel heat shock protein 70-like gene, *pss1+* of *Schizosaccharomyces pombe* homologous to Hsp110/Sse subfamily. *Gene*, **210**, 143-150.
- Ciocca, D.R., Clark, G.M., Tandon, A.K., Fuqua, S.A., Welch, W.J. and McGuire, W.L. (1993a) Heat shock protein hsp70 in patients with axillary lymph node-negative breast cancer: Prognostic implications. *J. Natl. Cancer Inst.*, **85**, 570-574.
- Ciocca, D.R., Oesterreich, S., Chamness, G.C., McGuire, W.L. and Fuqua, S.A. (1993b) Biological and clinical implications of heat shock protein 27,000 (hsp27): A review. *J. Natl. Cancer Inst.*, **85**, 1558-1570.
- Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J.H., Gorwa, M.F., Colavizza, D. and Thevelein, J.M. (1998) Involvement of distinct G-