Published in final edited form as:

Curr Genet. 2019 October ; 65(5): 1127–1134. doi:10.1007/s00294-019-00978-8.

Not quite the SSame: unique roles for the yeast cytosolic Hsp70s

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Abstract

The Heat Shock Protein 70s (Hsp70s) are an essential family of proteins involved in folding of new proteins and triaging of damaged proteins for proteasomal-mediated degradation. They are highly conserved in all organisms, with each organism possessing multiple highly similar Hsp70 variants (isoforms). These isoforms have been previously thought to be identical in function differing only in their spatio-temporal expression pattern. The model organism *Saccharomyces cerevisiae* (baker's yeast) expresses four Hsp70 isoforms Ssa1, 2, 3 and 4. Here, we review recent findings that suggest that despite their similarity, Ssa isoforms may have unique cellular functions.

Keywords

Chaperone; Hsp70; Ssa; Isoform; Evolution

Introduction

The Heat Shock Protein 70 (Hsp70) family of proteins are responsible for the folding of both newly synthesized and stress-damaged protein “clients” (Craig and Marszalek 2017; Kim et al. 2013; Nillegoda et al. 2018; Hubscher et al. 2017). Collectively these proteins are essential for viability and are highly conserved, following the same two domain architecture; a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD) connected via a flexible linker region (Kampinga and Craig 2011; Mayer 2018; Mayer and Bukau 2005). The NBD binds and hydrolyzes ATP to ADP, a process which promotes large-scale conformational change in the SBD, initiating client folding and release (Kampinga and Craig 2011; Mayer 2018; Mayer and Bukau 2005). Hsp70s do not function in isolation; their function and specificity are dictated by a suite of co-chaperones consisting of J-proteins and nucleotide exchange factors (NEFs) (Craig and Marszalek 2017; Nillegoda et al. 2018;

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Walsh et al. 2004). Co-chaperones function to accelerate the ATPase activity of Hsp70 and also directly bind and transport clients to the SBD for refolding (Craig and Marszalek 2017; Nillegoda et al. 2018; Walsh et al. 2004).

Hsp70 function has been extensively studied in the model organism *Saccharomyces cerevisiae* (budding yeast). Yeast encode seven cytosolic Hsp70s [Ssa1–4, Ssb1–2 and Ssz1 (Boorstein et al. 1994; Kabani and Martineau 2008)]. In addition, there are three mitochondria-specific isoforms (Ssc1, Ssq1 and Ecm10) and one specific to the ER (Kar2). The Ssa1–4 proteins arose from genome duplication and are highly conserved, with Ssa1 sharing 99%, 84% and 85% amino acid identity with Ssa2, 3 and 4, respectively (Fig. 1a). The highest sequence variation occurs within the SBD, specifically the outer-facing region of the “lid” (Fig. 1b, red highlighted region). While the lack of conservation of sequence in this area might suggest a low functional importance, a more likely scenario is that it dictates the nature of client proteins that bind to each Ssa isoform. Interestingly, a second area that also displays variation is in the NBD (Fig. 1b, orange highlighted region). This surface has been defined as a region for interaction with J-protein co-chaperones and may imply variation in the complement of co-chaperones that interact with each Ssa isoform (Craig and Marszalek 2017; Nillegoda et al. 2018; Walsh et al. 2004).

Despite sequence similarity, the Ssa1–4 proteins differ substantially in expression level in the cell. Ssa1/2 are expressed constitutively at high levels whereas Ssa3/4 are only expressed during cell stress (Werner-Washburne et al. 1987,1989; Boorstein and Craig 1990a,b; Werner-Washburne and Craig 1989). Studies of protein stability have identified significant differences in Ssa isoform half-lives; 20.2 h for Ssa1, 14.9 h for Ssa2, 11.0 h for Ssa3 and greater than 100 h for Ssa4 (Christiano et al. 2014). Cells lacking Ssa1/2 show dramatic upregulation of Ssa3/4 levels as a compensatory mechanism and cells overexpressing Ssa1 but lacking Ssa2–4 are fully viable and have no significant phenotypes (Werner-Washburne et al. 1987,1989; Boorstein and Craig, 1990a,b; Werner-Washburne and Craig 1989). While historically these proteins have been considered identical in function, recent findings suggest they may regulate unique facets of cell function.

Methodologies to understand redundant and non-redundant functions of Ssa proteins

Initial studies on Ssa function used cells lacking individual Ssas, possible due to the relative ease of PCR-based gene knockout (Gardner and Jaspersen 2014). These studies were complicated by the partial redundancy of Ssa1–4 and compensatory expression of Ssa3 and 4 in cells lacking Ssa1 and 2. Follow-up studies creatively utilized strains lacking Ssa1–3 while expressing a temperature-sensitive version of Ssa1, *ssa1–45* (Becker et al. 1996). The *ssa1–45* mutant protein possesses a single-point mutation at P417L which alters SBD structure and loses function at elevated temperatures allowing study of client response to Hsp70 inhibition. More recently, researchers have been aided by the creation of *ssa1–4* yeast strains in which all 4 genomic Ssa genes have been removed; these are kept viable by expression of Ssa1 from a *URA3*-based plasmid (Jung et al. 2000; Jaiswal et al. 2011). These strains can be transformed with plasmids expressing Ssa isoforms, Ssa1 point

mutations or Hsp70s from other organisms (Jung et al. 2000; Jaiswal et al. 2011). Curing of these strains on 5-fluoroorotic acid containing media promotes loss of the original Ssa1 plasmid, leaving cells expressing the desired Hsp70 variant as the sole cytosolic Hsp70 (Jung et al. 2000; Jaiswal et al. 2011). This newer system offers several advantages over *ssa1-45*. In the newer *ssa1-4* the exogenous Hsp70 expressed is the sole cytosolic Hsp70 in the cell, as opposed to the present (but inactive Ssa1) in *ssa1-45* cells. In addition, *ssa1-45* cells must be incubated at elevated temperature to suppress native Ssa1 function, non-ideal when studying regulation of the heat shock response.

Early indications of functional differences in Ssa isoforms

Initial indications of a functional difference in Ssas came from in vitro studies attempting to understand the role of Hsp70 in uncoating bovine brain-coated vesicles (Gao et al. 1991). Despite the ability of Ssa1,2 and 4 to bind coated vesicles (Ssa3 was not examined), Ssa1 displayed considerably less uncoating activity than the other two isoforms (Gao et al. 1991). Nearly a decade later, researchers identified a requirement for Ssa2 in the import of yeast Fructose 1,6-biphosphatase, FBP1 into similar vesicles (Brown et al. 2000). Lysate prepared from *ssa2* cells do not support the import FBP1 into vid (vacuole import and degradation) vesicles and FBP1 becomes stabilized in *ssa2* cells (Brown et al. 2000). Follow-up studies confirmed that Ssa2 was indeed critical in Vid pathway function, and that the specificity of this function came from a single amino acid difference between Ssa1 and Ssa2; A83 in Ssa1, G83 in Ssa2 (Sharma and Masison 2011).

Genetic and physical interactions of the Ssa proteins

Proteins have unique physical and genetic interactions; comprehensive analysis of this interaction “fingerprint” can provide useful insight into their specific function and even where a protein resides in the cell (Huang et al. 2002; Kuzmin et al. 2018; van Leeuwen et al. 2016, 2017). Analysis of currently known physical interactors of each Ssa isoform reveals substantial differences in interactomes sizes (Fig. 2a). The constitutively expressed Ssa1 and 2 have much larger interactomes compared to inducible Ssa3 and 4 (717 and 375 proteins, respectively, vs 69 and 57). This result is unsurprising given the relative expression of these isoforms under the conditions these studies were carried out (30 °C in mid-log phase). It is possible that Ssa3 and Ssa4 have the ability to bind some of the Ssa1 and Ssa 2 interactors, but never have the chance due to low stoichiometry. Yeast respond to high-temperature stress by inducing a large number of genes (mostly directed by Hsf1, see below). It is feasible that Ssa3 and Ssa4 have unique heat-induced interactors only seen at high temperature. While there are some shared physical interactors between Ssa1–4, they only make up 1.5% of the total interactions which is curious given their amino acid similarity. Proteins tend to be identified as client proteins of the Hsp70 isoforms on an individual basis as researchers discover their protein of interest has Hsp70-binding properties. To date, few attempts have been made to isolate the full complement of Hsp70 interactors, essential for a greater understanding of global Hsp70 function. Several groups, including ours have recently utilized affinity purification followed by mass spectrometry (AP-MS) to uncover the global interactomes of Hsp70 and Hsp90 chaperones under a variety of conditions and genetic backgrounds (Dushukyan et al. 2017; Woodford et al.

2016; Wolfgeher et al. 2015; Truman et al. 2012, 2015a, b; Dunn et al. 2015; Mollapour et al. 2011). At this time, no attempt has been made to characterize all the individual Ssa isoform interactomes, with only Ssa1 being examined in this fashion (Truman et al. 2012, 2015a, b). Proteomic analysis of Ssa1 interactomes under a range of physiological conditions will provide a greater insight into possible client interactions.

Genetic interactions between pairs of genes have long been used in yeast to understand unique and redundant gene function. However, large-scale screens in yeast utilizing the yeast knockout gene collections, pioneered by the Andrews and Contanzo groups have provided unparalleled mapping of yeast pathway control (Huang et al. 2002; Kuzmin et al. 2018; van Leeuwen et al. 2016, 2017). Analysis of the genetic interactors of Ssa1–4 reveal a very different picture than portrayed by the physical interaction data. The Ssas have almost no overlap in genetic interactors, even between pairs of constitutive Ssas (Ssa1 and 2) or inducible forms (Ssa3 and 4) (Fig. 2b). In addition, Ssa3 has almost the same number of unique genetic interactors as Ssa1 and 2, surprising given the low expression of Ssa3 at 30 °C and the very mild phenotype exhibited by *ssa3* cells. Future analyses combining gene pairs and cell stresses may provide greater insight into the unique functions of Ssa3 and Ssa4.

The potential for both overlapping and unique functionality amongst the Ssa family was further highlighted through global gene-expression analysis of yeast cells harboring Ssa1, 2, 3 or 4 as the sole Ssa in the cell (Hasin et al. 2014). While a wide variety of genes encoding for proteins in different cellular metabolic pathways were found to share similar expression profiles across the Ssa family, there was a large number of apparently Ssa-specific genes that showed altered regulation (Hasin et al. 2014). To fully appreciate the global cellular significance of each individual Ssa, a comprehensive meta-analysis of all phenotypic, genetic and molecular system-level data needs to be carried out.

Regulation of the heat shock response

In response to heat stress, cells must produce a variety of chaperones that include Hsp70 and Hsp90 that are capable of refolding denatured proteins. (Craig and Marszalek 2017; Kim et al. 2013; Nillegoda et al. 2018). This heat shock response is mediated through a complex feedback mechanism between chaperones and Heat Shock Factor 1 (Hsf1) (Koike et al. 2018; Truman et al. 2007; Verghese et al. 2012). In unstressed cells, Hsf1 is sequestered in a monomeric inactive form. Upon heat shock these chaperones become titrated away from Hsf1, binding to the increased number of unfolded proteins. At this point, Hsf1 is free to trimerize, bind to DNA and activate transcription of a large number of genes that includes chaperones themselves (Verghese et al. 2012). As the proportion of unfolded proteins decreases, chaperones become free to re-bind and re-inhibit Hsf1 function, resetting the cell to its basal state (Verghese et al. 2012). In yeast, the deletion of Ssa1 and Ssa2 (constitutive cytosolic Hsp70s) leads to activation of Hsf1 even under normal temperatures. *ssa1 ssa2* cells acquire thermotolerance at temperatures as low as 23 °C (Verghese et al. 2012; Matsumoto et al. 2005). This anomaly is due to the overexpression of other Hsps (including Ssa4) that occur upon loss of Ssa1/2. Yeast lacking the Hsp70 nucleotide exchange factors Sse1 and Fes1 possess constitutive Hsf1 activation, suggesting that the ATPase activity of

Hsp70 is key in Hsf1 regulation (Gowda et al. 2016; Liu et al. 1999). Consistent with its role in the heat shock response, cells expressing Ssa3 as the sole Ssa isoform are significantly more thermo-tolerant, a role that is interestingly independent of Hsp104 (Hasin et al. 2014).

Differential control of the heat shock response by specific Hsp70 isoforms has been observed in other species. The starlet sea anemone *Nematostella vectensis* expresses 3 main cytosolic Hsp70 homologs, NvHsp70A, B and D. Expression of these isoforms parallels the expression patterns seen in yeast, where NvHsp70D is constitutively expressed in contrast to the stress-inducible NvHsp70A and B isoforms (Waller et al. 2018). Given their high homology to Ssa1–4, it is unsurprising that NvHsp70A, B and D are capable of providing essential cell function in yeast lacking Ssa1–4 (Waller et al. 2018). What is more interesting is that yeast solely expressing NvHsp70B from a constitutive promoter are generally more stress resistant than cells expressing the other *Nematostella* isoforms (Waller et al. 2018). Global interactome analysis of these three isoforms when expressed in yeast reveal substantial client and co-chaperone specificities (Truman, unpublished data).

Hsp70 isoform-specific regulation of protein refolding and aggregation

Yeast possess several naturally occurring prions, the most well studied of which are [*PSI*⁺] and [*URE3*] which arise, respectively, from transformation of the Sup35 and Ure2 proteins. The propagation and aggregation of these prions is dependent on protein-folding machinery of the cell that include Hsp70, Hsp90 and Hsp104 (Jung et al. 2000; Guinan and Jones 2009; Jones et al. 2004; Jones and Masison 2003; Loovers et al. 2007; Matveenko et al. 2018). Screening of randomly mutagenized Ssa1 and Ssa2 identified several key residues required for prion propagation, the majority of which resided in the N-terminal ATPase domain (Loovers et al. 2007). There is a clear functional distinction between Ssa1 and Ssa2 in regards to prion propagation; overexpression of Ssa1 (but not Ssa2) promotes loss of [*URE3*] (Schwimmer and Masison 2002). In contrast, mutation of Ssa2 but not Ssa1 impairs [*URE3*] propagation (Roberts et al. 2004). Experiments with Ssa1–Ssa2 chimeras allowed identification of a single residue (glycine 83) in Ssa2 responsible for the stable Ssa2p-like [*URE3*] phenotype. This result is unlikely to be explained by the minimal differences in the intrinsic ATPase activities of Ssa1 and Ssa2; while never tested the authors raise the interesting possibility that the phenotype produced by this mutation (and observable differences between Ssa1 and Ssa2) might arise as a result of altered co-chaperone binding (Sharma and Masison 2011).

The Hsp70s have been identified as regulators of proteins responsible for human pathologies (Brehme and Voisine 2016). Several examples include CFTR in cystic fibrosis, SOD1 in ALS, Tau protein in Alzheimer's disease and Huntingtin protein in Huntington's disease (Brehme and Voisine 2016). While Hsp70 has been identified as binding and regulating these novel disease client proteins, historically there has been little study on the relative contribution each Hsp70 isoform makes in this role.

Excitingly, a recent study demonstrates that Ssa isoforms in yeast differentially promote degradation of α -synuclein through autophagy (Gupta et al. 2018). Hsp70 and other chaperones such as Hsp90 and Hsp104 interact with α -synuclein and in turn inhibit its

ability to form amyloid fibrils (Gupta et al. 2018). In contrast to prion propagation, cells expressing only Ssa3 isoform were the most resistant to α -synuclein toxicity. Studies of Ssa2–Ssa3 chimeras pinpointed a key role of the Ssa3 NBD in suppression of α -synuclein toxicity. The reason for this selectivity remains undetermined, although the authors of the study ruled out differences in Hsp90/Hsp104 activity in Ssa3 vs Ssa2-expressing yeast (Gupta et al. 2018).

Regulation of yeast cyclins

Cell cycle progression in yeast and mammalian cells is tightly regulated via the stability of Cyclin D1 (Cln3 in yeast). Cln3 degradation can be promoted by Cdc28-mediated phosphorylation of yeast Hsp70 (Truman et al. 2012). Given the importance of this mechanism to cell growth, it is unsurprising that when expressed at constitutive levels all of the Ssa isoforms can equally bind and regulate Cln3. Yeast undergo arrest in the G1 stage of the cell cycle upon treatment with alpha factor peptide pheromone. This process is controlled in a similar manner to above by an environmentally controlled CDK, Pho85 whose substrate specificity is determined through Pcl cyclin binding. While all Ssa isoforms bound Pho85 cyclins Pcl2 and Clg1, there was Ssa isoform-specific interaction with the other Pcls. Pcl6 bound Ssa1 and 2, Pcl7 bound uniquely to Ssa1 and Pcl8 bound to Ssa2, 3 and 4 [34]. This is a rare example of Ssa isoforms displaying client selectivity.

Specific post-translational modification of Hsp70 isoforms

The majority of research over the past few decades has focused on how Hsp70 function is regulated through transcription, expression of different isoforms and co-chaperone binding (Craig and Marszalek 2017; Kim et al. 2013; Nillegoda et al. 2018). Global proteomic analyses have uncovered a substantial number of modified sites on both Hsp70 and Hsp90 (Nitika and Truman 2017; Cloutier and Coulombe 2013). Phosphorylation can be added and removed rapidly, allowing fine-tuning of chaperone function when required. The large number of detected phosphorylations suggest a “chaperone code” similar in nature to the combinatorial PTM code that exists on histones (Nitika and Truman 2017; Cloutier and Coulombe 2013; Sager et al. 2018, 2019; Mollapour and Neckers 2012). Several regulatory phosphorylation sites have been detected on Hsp70 in human cells; these include T38, T66, T495, T504 and T636 which control cell cycle progression, condensation of chromosomes, translational inhibition, Hsp70 dimerization and client triaging, respectively (Nitika and Truman 2017). It is interesting to note that in all these cases, these sites are absolutely conserved in all Ssa isoforms (Fig. 1a). Mapping of phosphorylation sites previously detected in yeast global proteomic screens reveals that while phosphorylated sites appear to be highly conserved between Ssa1–4 (17/22 sites = 77%), there are several sites that are not. S530 and S535 are only present on Ssa1 and Ssa2, with these sites being replaced with the non-phosphorylatable residues alanine and asparagine, respectively, on Ssa3 and Ssa4 (Fig. 1a). It is interesting to speculate that the variation in these sites alters client specificity and may contribute to the functional differences between the inducible and non-inducible Ssas. In particular, we envisage a scenario where the function of constitutively highly expressed Hsp70 isoforms can be quickly switched by altering the Hsp70 chaperone code. This would

be unnecessary for the stress-inducible Ssas whose function is presumably suited to the stresses under which they are induced.

Conclusion and perspectives

Molecular chaperones have been studied for over 50 years, but the question remains why does a cell need to express so many Hsp70 variants? While research suggests they have greatly overlapping function, it is clear that they have unique roles that go beyond differences in transcriptional patterns. While small differences in Ssa ATPase activity might explain differences in refolding clients in vitro, they can-not account for the larger functional differences observed. A more likely explanation is that the individual isoforms in yeast (and other organisms) are unique in their co-chaperone and client binding (Fig. 3). The Ssa proteins are activated by a large number of J-protein and NEF co-chaperones; binding selectivity and affinity of these to each Ssa remains undetermined. Large-scale analysis of Hsp70 isoform interactomes by high-resolution mass spectrometry of the type seen in (Truman et al. 2012, 2015a, b; Knighton et al. 2019) would confirm that even the small amino acid differences present in Hsp70 isoforms can greatly impact co-chaperone and client-binding specificity.

Acknowledgements

This work was supported by NCI R15CA208773 (AWT).

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Fig. 1.
The Ssa proteins are highly conserved. **a** Alignment of amino acid sequences of *Saccharomyces cerevisiae* Hsp70 isoforms Ssa1, Ssa2, Ssa3 and Ssa4. Amino acids previously identified as being phosphorylated via mass spectrometry are annotated with a red dot. **b** Position of sequence variance between Ssa1–4 mapped onto the structure of Hsp70 (based on PDB entry 2KHO). Areas of clustered variance are enclosed in dashed red and orange circles

Fig. 2. Physical and genetic interactions of Ssa1–4. **a** Physical interactors of Ssa1–4 were obtained from the Saccharomyces genome database (<https://www.yeastgenome.org/>) and analyzed by Venn diagram using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>). **b** Genetic interactors of Ssa1–4 were obtained from the Saccharomyces genome database (<https://www.yeastgenome.org/>) and analyzed by Venn diagram using Venny 2.1 (<https://bioinfogp.cnb.csic.es/tools/venny/>)

Fig. 3.
Ssa isoform promoter and coding sequence may dictate functional specificity through a variety of mechanisms. Arrows represent possible connections between these features