Cell. Mol. Life Sci. 65 (2008) 855 – 861
1420-682X/08/060855-7
DOI 10.1007/s00018-008-7458-y

Cellular and Molecular Life Sciences

Visions & Reflections (Minireview)

New insights into the mechanism of heat shock response activation

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Received 3 October 2007; received after revision 8 December 2007; accepted 17 December 2007
Online First 1 February 2008

Abstract. Heat shock (HS) response is a universal mechanism of protection against adverse environmental conditions. It is manifested mainly by rapid and robust induction of molecular chaperones and other cytoprotective proteins. In higher eukaryotes the activation of the HS response is mediated by a master regulator, heat shock transcription factor 1 (HSF1). Here we outline recent progress in understanding the early steps in HSF1 activation by heat in the context of existing models of HSF1 regulation.

Keywords. Heat shock response, mechanism, molecular chaperones, heat shock transcription factor 1.

The heat shock (HS) response is one of the most evolutionarily conserved defensive mechanisms against acute exposure to extreme environmental and pathological conditions. Although many differences exist in the details, the general features of the HS response are remarkably conserved from bacteria through mammals. These include the rapid and massive increase in expression of HS genes. While some induction occurs due to altered mRNA stability, most regulation takes place at the level of transcription [1–5].

Recent whole genome analysis of gene expression patterns that occur in response to HS revealed an unexpectedly complex picture. Among the genes strongly induced by HS are chaperones, proteins that protect against oxidative damage, such as catalase and SOD, and other cytoprotective HS proteins (HSPs) that ultimately minimize the deleterious effects of stress, prevent apoptosis and ensure cell survival [6, 7].

Interestingly, the HS response is invoked by a wide range of diverse stressors, such as alcohols, hypoxia, transition metal ions, peroxide, amino acid analogs, etc. Many of these stressors are believed to cause the accumulation of abnormal proteins, which is, therefore, postulated to be the key factor in inducing the HS response [8, 9]. Fluidity and other physical characteristics of the cell membrane were also tied to the determination of HS response threshold temperature [10].

In eukaryotes, induction of the HS response is controlled by HS transcription factors (HSFs). HSF1 is constitutively expressed in higher eukaryotes and becomes rapidly activated post-translationally upon exposure of cells to elevated temperature [11, 12]. HSF1 is a member of the family of "winged" helix-turn-helix (HNF-3/fork) transcription factors [13, 14]. A single HSF protein is expressed in lower eukaryotes, such as yeast [15], *Drosophila* [16] and *Caenorhabditis elegans* [17]. However, *Xenopus laevis* expresses two distinct HSFs [18, 19], mammalian cells express at least three HSFs [20, 21], and plants express multiple (more than 20) HSF proteins [22]. One form of HSF,

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HSF3, has been identified only in avian cells [23]. An overwhelming body of evidence suggests that HSF1 is the master regulator of heat-inducible HSP gene expression in vertebrates. Initial data suggested that HSF2 was involved mainly in development [24, 25], but recent reports show that it may also participate cooperatively with HSF1 in stress-induced activation of HS genes [26]. While HSFs from different species share relatively little homology, the overall domain organization of HSF1 is conserved. Briefly, the N-terminal DNA binding domain of HSF is followed by two hydrophobic repeat domains (HR-A/B) and by a loosely defined regulatory domain. An additional hydrophobic repeat, HR-C, is situated further downstream and followed by a transactivation domain. Yeast *Saccharomyces cerevisiae* HSF contains the essential constitutive transactivation domain and an additional activation domain, which is heat-activated. The high homology between the DNA binding, HR-A/B, and HR-C domains in different organisms is remarkable [27].

In a cell exposed to stress, HSF1 is rapidly converted to its active form. The activation event is associated with the transition of the monomer to a trimer [12, 28]. Trimerization occurs *via* intermolecular coiled-coil interaction of the HR-A/B and HR-C domains [29]. In turn, the trimeric form of HSF1 possesses high affinity for its cognate DNA consensus sequence present in most heat-inducible promoters. This sequence (HSE, heat shock element) consists of repeating five-nucleotide units of nGAAn in head-to-tail orientation [30]. The minimal sequence required for HSF1 trimer binding contains two HSE elements; three elements allow formation of the strongest HSF-DNA complex. Binding of monomeric HSF to HSE has not been described. In *S. cerevisiae*, HSF is constitutively localized to the nucleus and bound to HSE, although recent reports suggest an increase in the DNA-binding level in response to HS. Still, the bulk of HS response regulation in yeast occurs at the level of transactivation ability of yeast HSF, which is strongly induced by HS. In vertebrate cells, HSF1 activation occurs concomitantly with its re-localization to the nucleus [12]. Interestingly, in human, but not in murine, cells, HSF1 forms characteristic discreet subnuclear structures of unknown physiological function, called stress granules [31, 32]. These structures appear within seconds of exposure to stress and include a number of RNA-binding proteins. Stress granules were observed to be localized to large regions of heterochromatin on chromosomes 9, 12, and 15 [33].

In addition to trimerization, HSF1 undergoes other post-translational modifications, such as phosphorylation and SUMOylation [34]. Stress-induced phosphorylation was described for at least 12 Ser residues. Curiously, no Thr or Tyr residues have been found to be phosphorylated. Ser phosphorylation, by contrast, increases dramatically with exposure to HS. Recently, a systematic study of heat-induced Ser phosphorylation of HSF1 was performed [35]. While the majority of 12 phosphorylated Ser residues have not been ascribed any physiological function, phosphorylation of Ser 326, Ser303 and Ser307 appeared to correlate with the activation of the factor's transcriptional competence. Interestingly, earlier reports suggested that Ser303 and Ser307 phosphorylation occurs during de-activation of HSF1, after HS. SUMOylation – a covalent attachment of a short, ubiquitin-like peptide to a specific Lys residue (Lys298) – requires prior phosphorylation of the nearby Ser303 residue [36]. Although the role of SUMOylation in HSF1 regulation is obscure, some reports suggest it modulates HSF1 DNA binding [37] and transcriptional activity [38].

Soon after HSF was discovered, it became apparent that it exists in the cell under tight negative regulation. Several lines of evidence suggested the existence of a titratable cellular factor that inhibits HSF under normal conditions. For example, overexpression of HSF in cell lines inevitably resulted in constitutive activation of the factor, as did expression of recombinant protein in bacteria [4]. Heterologous expression of *Drosophila* HSF in human cells also led to constitutive activation, while expression of human HSF1 in *Drosophila* cells lowered the temperature threshold of HSF1 activation by ~10 degrees, to the temperature that induces the *Drosophila* HS response [39].

To explain these data, a model of HSF regulation by a negative feedback mechanism involving an HSP70/HSP40 complex was proposed, based largely on the biochemical identification of protein-protein interactions [40–42]. Initially, monomeric HSF was thought to be stabilized under normal conditions by interaction with HSP70/HSP40. During HS, HSP70/HSP40 was thought to interact with accumulating denatured and partially unfolded protein, thereby releasing HSF and relieving its repression. Once dissociated from the inhibitory complex, HSF trimerized spontaneously and acquired DNA-binding ability, leading to the production of increased amounts of HSP70 and HSP40. After sufficient amounts of these chaperones were synthesized to bind all available non-native proteins, excess HSP70 would bind to HSF trimers causing them to dissociate and revert to the inactive, monomeric state [43, 44]. Subsequently, interaction of HSP70 with HSF was reported to occur in both unstressed and heat-shocked cells and was shown to be insufficient to repress HSF under normal conditions [45]. Other reports indicated that this interaction

did play a role in the repression of the ability of HSF to transactivate transcription, but that it was unrelated to the regulation of the ability of HSF to bind DNA [44]. The enormous molar excess of intracellular HSP70 over HSF should make any fine-tuned negative regulation very difficult, if not impossible, especially since the HSP70 family of chaperones displays relatively low substrate specificity and the HS-inducible and constitutive forms of HSP70 are closely related.

HSP90, another ubiquitous and highly abundant chaperone, was next postulated to be the specific HSF repressor. HSP90 was shown to be involved in the regulation of many diverse signaling proteins, such as steroid hormone receptors, kinases, transcription factors, and others, by forming a multichaperone complexes with client proteins [46]. Such a complex typically forms *via* several successive steps involving HIP, HOP, HSP70/40, p23, and immunophilin, before maturing into its final form. Highly specific inhibitor of HSP90 geldanamycin and its derivatives cause activation of the HS response, including HSF1 trimerization and binding to DNA in variety of organisms and cell lines. Again, HSF trimerization was suggested to occur spontaneously after HS induced dissociation of the inhibitory complex [47].

The yeast two-hybrid protein interaction system was used to identify HSFBP1, a small HSF1-binding protein that inhibits HS promoter activation *in vivo*, and that has been suggested to negatively affect the DNA-binding activity of HSF [48]. HSFBP is a small evolutionary conserved protein that contains two hydrophobic heptad repeat regions similar to those found in HSF. Interestingly, it appears to exist as a trimer over a wide range of concentrations *in vitro*. Because of the intrinsic features of the two-hybrid system, it should preferentially yield targets that affect the transactivation capacity of HSF. Indeed, another protein identified as an HSF partner by this method is DAXX [49]. It was shown to enhance HSF transactivation function, contrary to its other role as a repressor of basal transcription. Finally, overexpression of the HSP90 and HSC70 co-chaperone, CHIP, was reported to induce HSF1 activation in murine fibroblasts [50], accompanied by a nearly quantitative trimerization of HSF1 and, remarkably, by formation of a complex containing HSP70. Because CHIP also functions as a ubiquitin-ligase, this mechanism is likely to be involved in the fine regulation of the folding/proteasome degradation capacity of the cell [51].

The induction of HSF by a variety of diverse stressors suggests that HSF may be a convergence point for several signaling pathways. Non-native proteins have been proposed to be a shared characteristic of all HSF-activating stresses [8, 9]. Alternatively, it was suggested that HSF is able to sense directly various kinds of stresses, such as heat, peroxide, and low pH [52, 53]. Indeed, purified HSF from *Drosophila* was activated *in vitro* by exposure to each of these agents. However, a high (micromolar) concentration HSF was required and reversal of its activation did not take place until it was diluted sufficiently. Similar results were reported for mouse HSF1, either translated *in vitro* in reticulocyte lysate or expressed in *E. coli* [54, 55]. The *in vitro* trimerization of HSF1 in response to heat and oxidative stress was shown to be dependent on two Cys residues located within DNA-binding domain. Mutation of these cysteines to serine rendered the factor unable to undergo trimerization and nuclear translocation *in vivo* [56]. Since mutations in hydrophobic repeat domains HR-A, B and -C abrogated the observed HSF1 activation, an intramolecular repression mechanism seems to be necessary to maintain HSF in the repressed state under normal conditions [57, 58]. However, intramolecular repression and spontaneous trimerization alone cannot adequately explain all features of the HSF activation process, especially its early steps.

As mentioned above, the activation temperature of HSF mimics that of its host during heterologous expression [39]. This strongly suggests the presence of auxiliary factors that determine the precise conditions of its activation and that possibly actively participate in the process itself. Furthermore, any mechanism that involves competition between HSF and exposed hydrophobic regions of denatured proteins for HSP70 interaction would, considering the abundance of HSP70 in the cell, require a massive denaturation of cellular proteins within seconds of shift to high temperature. The attenuation of HSF activity would then require *de novo* synthesis of HSPs to saturate the available binding sites before effective repression of HSF could be reestablished. This process would take hours, and yet, HSF granules – subnuclear structures that are the hallmark of the HS response and thought to be composed largely of active HSF – appear within seconds of exposure to high temperature and disappear rapidly upon return to the normal growth temperature [32].

Despite numerous studies and extensive characterization, no defined *in vitro* system has been developed that reproduces HSP90-mediated repression of HSF *in vitro*. Arguably, this could be due to the dynamic nature of inhibitory complex formation *in vivo* [27]. Alternatively, additional factors may be required for the efficient activation of HSF to occur, even after it has been released from its complex with HSP90. In the absence of the reconstituted *in vitro* system for HSF1 repression by the HSP90 complex, it is difficult to attribute the effect of geldanamycin and other HSP90

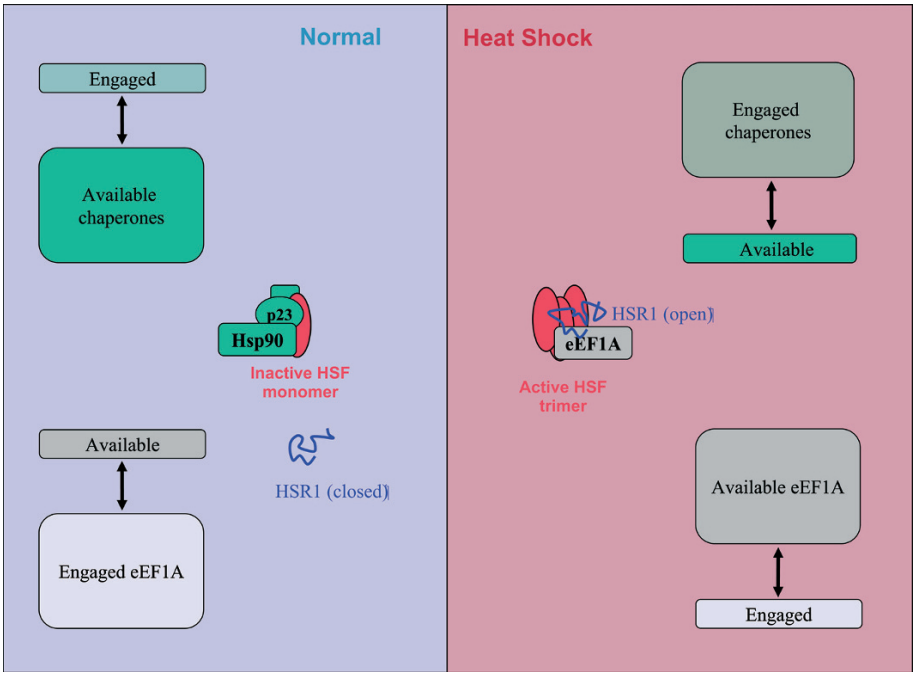

Figure 1. Integrated model of heat shock transcription factor 1 (HSF1) activation control. Under normal conditions (left panel) the bulk of eEF1A is engaged in translation and maintenance of the cytoskeleton. At the same inactive HSF1 monomer is sequestered in the inhibitory complex with abundant HSP90 and co-chaperones. HSR1 is present in inactive "closed" conformation. During HS (right panel) HSR1 "switches" into the "open", HSF1-activating conformation, while massive release of elongation factor eEF1A occurs due to translation shutdown and cytoskeleton collapse. Accumulation of denatured and misfolded cellular proteins depletes the pool of chaperones available for the assembly of HSF1 inhibitory complex. The formation of active HSF1 trimer is either directly promoted by eEF1A and HSR1 or is favored by the stabilizing interaction with them.

inhibitors specifically to the regulation of HSF1. Indeed, these drugs target and disrupt HSP90 interaction with many client proteins, which, inevitably, will spell a major perturbation to cell protein homeostasis. This alone may be sufficient to cause HSF1 activation in a manner similar to the HS response induction by proteasome inhibitors.

In summary, although the formation of the HSP90 multichaperone complex with HSF and its susceptibility to HS have been well-established and corroborated by several lines of evidence, it is unlikely to be the sole mechanism responsible for the regulation of HSF activation. Rather, it could be an important mechanism to sequester inactive HSF monomers to ensure that no HSF activation occurs under normal conditions. This would be consistent with the hypothesis that HSP90 may actually be a "molecular buffer" that maintains the general balance and fidelity of cellular signaling networks [59]. On the contrary, however, it appears that some HSF molecules exist in a complex with HSP70/HSP40. Such complexes may contain monomers under normal conditions, perhaps as intermediates during the formation of HSP90-containing inhibitory complexes or trimers during HS. This is not surprising given the difference in the relative abundances of the two proteins, the high propensity of HSP70 to interact with hydrophobic substrates and the existence of three hydrophobic repeat domains within the HSF molecule. However, the interaction of HSF with HSP70/HSP40 *per se* is unlikely to be the major regulatory mechanism.

Additional mechanisms of HSF regulation are becoming known. Translation elongation factor eEF1A and a novel non-coding RNA, HSR1, act in tandem to activate HSF during HS [60]. Curiously, eEF1A is one of the most conserved and most abundant proteins in eukaryotic cell. It has been implicated in a variety of cellular processes in addition to its canonical role in mRNA translation during protein synthesis [61]. Notably, eEF1A is the key component regulating the actin cytoskeleton architecture in the cell [62]. HSR1 is a novel, large, non-coding RNA that appears to be highly conserved. Computer modeling reveals that it possesses extensive secondary structure that is predicted to change within the physiological range of temperatures (I.S. and E.N., unpublished observations). Cells depleted of HSR1 by either transient or stable transfection with RNAi-expressing constructs display a severely impaired HS response. Together, purified HSR1 and eEF1A are capable of activating HSF1 *in vitro* at physiological concentrations. Moreover, this *in vitro* activation can be induced by a

physiologically relevant temperature increase (I.S. and E.N., unpublished observations). As a result, it is tempting to propose that HSR1 is the cellular thermosensor and that changes in its secondary structure occur in response to changes in temperature. The concept of an RNA thermometer is not new – a few bacterial examples have been described, although with completely different mechanism of action [63, 64].

Among its non-canonical roles, the participation of eEF1A in cytoskeleton organization is noteworthy because it provides an intriguing link between two fundamental cell functions and the HS response. The general shutdown of protein synthesis [65] and the collapse of the cytoskeleton [66] during HS release large amounts of free eEF1A that become available for interaction with HSF1. Although the kinetics of these processes is slower than that of HSF1 activation, given the high abundance of the cytoskeleton components and the limiting amounts of HSF1, this should be sufficient to trigger HSF1 activation. The same reasoning applies in the case of translational shutdown. The resulting large molar excess of eEF1A over HSF allows for instantaneous activation of HSF early during HS (Fig. 1).

The discovery of HSR1 and its role in the regulation of the HS response provides an exciting opportunity for the development of novel therapeutic treatments for cancer. In many tumors, elevated levels of HSPs are correlated with high metastatic potential and poor survival prognosis. Treatments that suppress the expression of HSPs in tumor cells may sensitize them to existing therapy. HSF1 has been successfully targeted in tumor cells to render them thermosensitive and susceptible to other chemotherapeutic treatments [67]. Although currently nothing is known about HSR1 expression levels and/or altered properties in tumor cells, the direct involvement in the HSF activation makes HSR1 an attractive therapeutic target.

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