Mini-Review

Heat Shock Proteins: The Search for Functions

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They sought it with thimbles, they sought it with care;
They pursued it with forks and hopes (6)

A Definition and History

Not so long ago, in the olden (golden ?) days, scientists interested in the biochemistry and cell biology of proteins attempted to explain an enzyme's catalytic and regulatory functions and the cytoskeleton's properties in terms of protein structure. But SDS PAGE, monoclonal antibodies, and cDNA libraries (nucleic acid sequences reverse transcribed from mRNAs and inserted into the genomes of bacteriophage or plasmids) changed things, and many of us now study proteins the other way around. We know structure, in the form of subunit molecular weights, isoelectric points, modifications by phosphate, sugars, methylations, etc., and in some cases, even primary sequences and the precise localization in the cell or tissue. What we search for is function. Such a search has been in progress over the past several years to discover what heat shock proteins (HSPs)[1] do. Here, I define an HSP by two criteria: (*a*) its synthesis is strongly stimulated by an environmental stress, in particular, that resulting from a change in temperature a few degrees centigrade above the normal physiological one, and (*b*) its gene contains a conserved sequence of 14 base pairs in the 5′ noncoding region, the Pelham box (62). This sequence serves as the promoter for HSP mRNA transcription.

The response of cells to a heat shock was first described about 25 years ago during a study of temperature effects on *Drosophila* embryos (65). A dramatic change was seen in the puffing pattern of polytene chromosomes in salivary glands, and this was later shown to be the result of very active gene transcription that led to the synthesis of a small set of proteins. For about 15 years, this selective induction of proteins by a heat shock was thought to be unique to the fly. In 1978, however, an analogous response in avian and mammalian tissue culture cells to heat shock was discovered in my laboratory (33), and others reported a similar activity in *E. coli* (39, 78) and *Tetrahymena* (21). Reports of proteins induced after heat shocking a variety of species quickly followed and we now recognize that virtually all organisms—from *E. coli* to man—have HSPs (see reference 68 for a more complete history). The major HSPs have been strongly conserved in structure through evolution, clearly indicating that they play a vital role in survival of the organism. Their presence appears to enhance the cell's ability to recover from stress but precisely how they do this is the question many of us are trying to answer.

In this brief report I review some of the data that provide clues for how four of the "universal" HSPs may function. Three of these are known only by their subunit molecular weights: HSP90, HSP70, and HSP 20–30. The precise molecular weight varies slightly among different organisms. In the eukaryotic cell, each group appears to constitute a gene family consisting of closely related protein isoforms and genes encoding sequences regulated by different promoters (14, 16, 19, 46, 50, 59, 79). The fourth HSP is ubiquitin, a protein of 8,000 mol wt. We know some functions for ubiquitin; these are described below. For a more comprehensive account of the heat shock phenomena, including the organization and regulation of expression of HSP genes, I recommend the recent excellent reviews by Craig (13), Lindquist (42), Neidhardt et al. (51), and Nover (54).

Approaches to the Problem

How does one identify a function? Two strategies come immediately to mind: (*a*) clone and sequence the genes encoding the proteins, and (*b*) isolate and characterize mutants carrying defects in the structural genes. Sequence homologies to proteins with known function could reveal the HSP's role. In fact, one HSP sequence, a stretch of 75 amino acids which are conserved among four small *Drosophila* HSPs, is 50% homologous to the B chain of mammalian lens alpha crystallin (32), suggesting that these HSPs may serve some kind of structural role. The complete amino acid sequences for HSP70 from *E. coli*, *S. cerevisiae*, *Drosophila*, and humans reveal very highly (>80%) conserved sequences throughout the polypeptide (2, 31, 42). Thus far, neither these sequences nor those known to be conserved between the yeast and *Drosophila* HSP90 (20) have been identified in sequences of the ~4,000 proteins in the data bank. Recently, a relationship between the sequences of HSP70 and a glucose-regulated protein, GRP78, was detected and the latter was shown to be identical to a protein that binds to the immunoglobulin heavy chain in pre-B cells (49).

The isolation of HSP mutants with identifiable phenotypes other than sensitivity to heat has provided some potentially important clues about the function of some HSPs. Mutants in HSP genes have been studied in both *E. coli* and *S. cerevisiae*. Many of the *E. coli* HSP mutants turned out to

1. *Abbreviation used in this paper*: HSPs, heat shock proteins.

be ones originally selected as bacterial genes involved in the replication of bacteriophages (23, 24). From studies of these mutants we know that the *E. coli* HSP70 is the product of the dnaK gene and interacts with lambda phage O and P proteins during phage replication (38). This interaction affects an ATPase activity of the HSP70 (see below for more about this activity). dnaK mutants are wild type in growth at 30°C but nonviable at 42°C. At the high temperatures, dnaK mutants accumulate large amounts of HSP70, probably because the mutationally altered protein cannot autoregulate its own synthesis (71), a property also of eukaryotic cell HSP70 (17). Another set of *E. coli* HSP mutants map in the groEL and groES genes which code for proteins that make unusual oligomeric structures and play a role in the assembly of phage heads and tails (29). The EL-ES proteins appear to be involved in septation and division of the normal cell. Mutations in another *E. coli* heat shock gene affect an ATP-dependent protease (9).

Yeast (*S. cerevisiae*) offers an important advantage for mutational analysis since it is possible in this organism to genetically engineer mutations in vitro in a known gene and simultaneously integrate the mutations into the yeast genome and delete the wild-type gene. Craig and co-workers have performed such acts with the eight genes making up the HSP70 family in yeast and obtained a variety of phenotypes that include sensitivity to cold, heat, and germination (15, 16, 42). A "knock out" of the heat-inducible yeast HSP90 gene revealed a second gene for this protein (20). Most puzzling of all are the results of deleting the gene for the yeast 26-kD HSP. This gene is activated during sporulation (35) as well as by heat but no effects of the loss of the gene on yeast growth, sporulation, spore stability, or germination were detected in the mutant (64). Perhaps other yeast proteins can compensate for the 26-kD protein in the manner by which *E. coli* groEL and ES mutants are suppressed by the lambda E gene, which codes for the major lambda head protein (23).

Another approach, currently in vogue, for identifying function uses monospecific antibodies. Both monoclonal and polyclonal antibodies now exist for each of the major HSPs from a variety of organisms. What they have revealed can be summarized as follows: (*a*) There is strong conservation in protein structure across widely divergent species for HSP70 and HSP90, but the smaller HSPs appear species specific. (*b*) Most tissues of an organism have constitutive or cognate forms of HSP70 and HSP90 with some variation in levels, e.g., in mammals, brain has particularly high levels of these proteins relative to liver. (*c*) HSP70 is mobile in the cell, moving from cytoplasm to nucleus very shortly upon a heat shock and then returning to the cytoplasm upon cell recovery; HSP70 concentrates in the nucleolus (11, 47, 63, 76). (*d*) HSP70 changes from a "soluble" cytosolic to a nuclear–cytoskeletal fraction after a heat shock, and this insoluble form is solubilized by an ATP-dependent reaction (11, 41). (*e*) The small HSPs form large insoluble aggregates in a perinuclear region of the cell after prolonged heat shock (11, 55), but these aggregates dissociate during cell recovery.

The "educated guess" should offer a more rational way for identifying function, but success requires knowing what happens in a cell after a heat shock. There is a complication here, for cells from different organisms respond differently depending on the extent of the temperature stress and even the same cell will exhibit different properties based on the degree of stress. For example, a heat shock of rat cells (76) or of barley aluerone (3) will collapse the cell's endoplasmic reticulum and Golgi stacks, as seen in electron microscopic images of thin sections. However, we found that a heat shock of chicken embryo fibroblasts that had been infected with vesicular stomatitis virus does not block the functions of the endoplasmic reticulum and the Golgi complex as measured by the synthesis and maturation of the virus' glycoprotein which is transported through these organelles (Schlesinger, M. J., and C. Malfer, unpublished experiments). The critical parameter that may produce these variations is the fractional increment of temperature over the normal physiological range, but there may be other factors such as cell growth rate, composition of the surrounding media, etc. Given this caveat, one finds a potpourri of data—too numerous to detail here—describing changes occurring in the heat-shocked cell (many are noted in references 13, 42, and 68). Among these are the cessation of DNA synthesis and condensation of chromatin (61), the dephosphorylation of ribosomal protein S6 (26, 56, 67), collapse of the intermediate filament network (11, 75), increases in intracellular calcium (18), shifts to glycolytic metabolism (28), etc. With such a broad assortment, it will be difficult to pinpoint where an HSP will function and an HSP70, for example, might have several sites for activity.

Then there is serendipity, "the gift of finding valuable or agreeable things not sought for." More often than most scientists care to admit, serendipity accounts for many important discoveries. The heat shock phenomena is no exception; many of us believe that the first reported observation of heat shock by F. Ritossa in *Drosophila* embryos was serendipitous. Fred Neidhardt notes that the key to regulation of the *E. coli* heat shock response was a "serendipitous mutant" (51). My own immersion into the heat shock problem could be considered the result of serendipity, for we were actually studying effects of amino acid analogues on the replication of a virus in chicken embryo fibroblasts when we discovered that these analogues induced a set of three cell proteins that turned out to be HSPs! A more recent example of how serendipity uncovered an activity of a mammalian HSP70 is worth recounting here. The story begins in June, 1983 when I was visiting Jim Rothman's lab and heard about a purified bovine protein that dissociated clathrin baskets in the presence of ATP. Many properties of this enzyme resembled those of a chicken HSP70 which Phil Kelley had purified in my laboratory, and we decided to test the bovine protein for reactivity to our anti-chicken HSP70 antibodies. To our surprise, a positive signal appeared in an immunoblot of the intact and proteolytic digestion products of the bovine clathrin uncoating enzyme. Further evidence was obtained by Bill Welch at Cold Spring Harbor Laboratory who noted that HSP70-like proteins have an ATP-binding property and found identity between proteolytic digestion products of HSP70-like proteins and the uncoding enzyme. Additional comparisons of this enzyme with HSP70s from yeast and mammalian sources in a collaborative effort among several labs studying heat shock confirmed that the protein that dissociates clathrin baskets in the presence of ATP is a member of the HSP70 gene family (8, 72). This identification of an interaction between HSP70 and a protein complex with ATP, leading to hydrolysis and dissociation, is reminiscent of two other similar reactions of an HSP70 in which the substrates were a lambda phage DNA replication complex (38, 80) and a

nucleolar or nuclear cytoskeleton fraction, possibly a ribonucleoprotein complex (41). Members of the HSP70 family have ATP-binding sites (8, 47, 74); they also bind fatty acids (27). All of this leads me to speculate that HSP70s participate in the "scaffolding" of protein complexes as well as in their dissociation, the latter driven by ATP hydrolysis.

Another set of circumstantial events has provided a potential clue to the function of HSP90. In 1980, Bishop and colleagues (57) were studying the cellular distribution of the Rous sarcoma virus pp60 kinase and noted that this enzyme was transiently associated with two cellular proteins, one of which had a molecular mass of 90 kD. In an adjacent laboratory, Levinson and Oppermann had been studying the effects of various thiol oxidants and heavy metals on chicken embryo fibroblasts and noted the induction of proteins resembling the HSPs (40). A comparison of the HSP90 and kinase-associated P90 by proteolytic digestion indicated the two were identical and this was confirmed when the P90 showed a positive response to our antibodies specific to the HSP90 (57). These results set the stage for the more recent revelations that HSP90 also forms a complex with steroid receptors (7, 66, 69). Both the receptor and kinase undergo intracellular translocations; in one case from cytoplasm to the nucleus and in the other (the kinase), from polysome to the cell's plasma membrane. The HSP90 could be involved in this transport or it could modulate these growth factor activities by forming inactive complexes.

Proteases and Heat Shock

Throughout the course of investigations on heat shock there have been speculations that a major consequence of heat shock on cells is a denaturation of proteins, followed by their degradation. In fact, the rather small increments in temperatures that trigger the heat shock response are insufficient to inactivate most enzymes or disrupt the conformation of structural proteins. Most attempts to measure the turnover of cellular proteins after heat shock fail to find increases in protein degradation, and one report shows a stabilization of proteins after heat shock (47). However, nascent polypeptides and partially folded subunits on their pathway to forming complex structures such as a ribosome might be sensitive to heat shock temperatures and they will be targets for proteases. In the eukaryotic cell there exists a major, universal, nonlysosomal, ATP-dependent system for degrading polypeptides. This pathway of proteolysis involves a small 76 amino acid polypeptide called ubiquitin (reviewed in reference 10). The name is appropriate for ubiquitin: it is present in all eukaryotes examined thus far and its amino acid sequence is invariant from flies to human. Yeast ubiquitin differs in only three of the 76 amino acids (58). Elegant studies on the ubiquitin system show that ubiquitin is activated by ATP at the carboxy-terminal glycine, followed by a series of enzymatic reactions that culminate in the attachment of ubiquitin to the ε-lysine groups on proteins. Polyubiquitinated proteins become targets for protease digestion; monoubiquinated histones H2A and H2B cycle between the free and modified forms.

The discovery by Ursula Bond in my laboratory that ubiquitin is an HSP did not come from a direct study of the ubiquitin pathway but instead from a survey of a cDNA library prepared from heat-shocked chicken embryo fibroblasts and screened by a differential colony hybridization (5). There had been two clues from earlier studies that suggested a role for ubiquitin in heat shock. Varshavsky and co-workers discovered that a temperature-sensitive mouse cell line was defective in the ubiquitin-activating enzyme and noted that these cells were unusually sensitive to a heat shock response (22). The second clue came from an unrelated study that showed that cells treated with amino acid analogues so that abnormal polypeptides were made had a 10-fold increase in the levels of ubiquitin-conjugated proteins (30). Amino acid analogues turn out to be good inducers of the heat shock response. In chicken embryo fibroblasts subjected to a heat shock, ubiquitin mRNA synthesis increases some fivefold and levels of free ubiquitin transiently rise (5). Ubiquitin mRNA levels also are elevated in the livers of mice and gerbils subjected to hyperthermia (Bond, U., T. S. Nowack Jr., and M. J. Schlesinger, unpublished data). There may well be other effects of heat shock on the ubiquitin system. For example, Glover noted a loss of ubiquinated histones after heat shocking *Drosophila* cells (26), and we have tentatively identified a ubiquinated histone as a protein that rapidly loses its ubiquitin when chicken fibroblasts are heat shocked (Bond, U., and M. J. Schlesinger, unpublished data).

The association between heat shock and proteases is found also in prokaroytes; one of the *E. coli* genes regulated by heat shock is lon which codes for an ATP-dependent protease (9). There is speculation that these protease activities may be part of the cell's system for sensing a heat shock stress and activating proteins that turn on the HSP genes (1, 48). All this is reminiscent of the proteolytic activities associated with lambda phage regulation and the SOS response to DNA damage in *E. coli* (43). We should know soon if the eukaryotic heat shock transcription factors are activated by factors in a proteolytic system; they have been partially purified and shown to function in vitro (60, 77). It should also be noted that the HSP70 itself is reported to have a protease activity (45).

Life Cycles and Heat Shock Proteins

Organisms can encounter wide fluctuations in temperatures of their surrounding throughout their life cycle. For example the bacterium *E. coli* cycles between the 37°C of the mammalian gut and the <20°C of waste water. Dimorphic fungi (e.g., *Histocapsulatum*) and the parasitic protozoa *Leishmania major* and *Trypanosoma brucei* experience similar temperature changes between hosts. Not surprisingly, these organisms induce HSPs when they initially encounter the higher temperatures (37, 73). Presumably the HSPs help the cell overcome temperature-induced damage in the same way they help other stressed cells whose life style shows less temperature variations. What is striking, however, and should be borne in mind when speculating about HSP function are the observations that several HSPs are induced during normal cellular development, in the absence of temperature stress (of course, there could be another kind of stress operating during these stages). During the sporulation of yeast, for example, genes for two of the major yeast HSPs are activated. The HSP70 gene itself is not activated under this condition but proteins for two HSP70 family members are induced (35). An HSP70-like protein is one of the first proteins synthesized after zygote formation in mouse embryogenesis (4),

and several HSPs are induced during oogenesis and pupation in *Drosophila* (25, 36, 44, 59, 70).

Virus Infection and HSP Synthesis

Bacteriophage lambda actually uses several of the *E. coli* HSPs for its replication (23). Several eukaryotic cell DNA viruses, i.e., adenovirus (52), herpes virus (53), simian virus 40, and polyoma viruses (34), activate synthesis of HSP70 early in infection. Newcastle disease virus, an RNA virus, induces HSP70 and HSP90 in infected chicken cells (12). As in the case of bacteriophage, the HSPs may be used by the virus replication system or their induction may simply reflect a stress from the infection. The fact that the adenovirus E1A gene product is an inducer of cell HSP70 argues for a positive role of this protein in the virus life cycle.

A Summing Up

Much of the data cited here suggest that the major HSPs perform primarily structural roles in contrast to catalytic activities. Our understanding of how they interact with existing cellular structures is slowly emerging, yet the ways that HSPs help a cell tolerate stress remain a mystery. Sorting out the existing clues and figuring out how to get more definitive ones to solve this mystery is where we are now. Shortly after I began studying HSPs, a colleague warned that I might be hunting the snark. Since then, many investigators have provided evidence to indicate that, at the least, this snark will not be so elusive. The search goes on. Surely, it will not be a trivial pursuit.

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