Mammalian HspB1 (Hsp27) is a molecular sensor linked to the physiology and environment of the cell

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Abstract Constitutively expressed small heat shock protein HspB1 regulates many fundamental cellular processes and plays major roles in many human pathological diseases. In that regard, this chaperone has a huge number of apparently unrelated functions that appear linked to its ability to recognize many client polypeptides that are subsequently modified in their activity and/or half-life. A major parameter to understand how HspB1 is dedicated to interact with particular clients in defined cellular conditions relates to its complex oligomerization and phosphorylation properties. Indeed, HspB1 structural organization displays dynamic and complex rearrangements in response to changes in the cellular environment or when the cell physiology is modified. These structural modifications probably reflect the formation of structural platforms aimed at recognizing specific client polypeptides. Here, I have reviewed data from the literature and re-analyzed my own studies to describe and discuss these fascinating changes in HspB1 structural organization.

Keywords HspB1 · Hsp27 · Dynamic oligomeric complexes · Phosphorylation · Cell environment · Cell physiology

Introduction

Human HspB1 (also called Hsp27 or Hsp28) is a true heat shock protein whose level is upregulated when cells are exposed to conditions that alter protein folding, such as heat shock. Together with HspB5 (alphaB-crystallin), HspB1 is probably the small heat shock protein that has been the most intensively studied. HspB1 was first purified and characterized in 1987–1988 (Arrigo et al. 1988; Arrigo and Welch 1987). It was immediately noticed that HspB1 had the intriguing ability to form high molecular weight oligomers and to display intense phosphorylation. Several years later, it was discovered that HspB1 is an ATP-independent molecular chaperone involved in the protein refolding machinery (Jakob et al. 1993). Its major role is to trap and store stress-induced mis-folded polypeptides to avoid their aggregation and to indirectly promote their refolding or proteolytic degradation (Ehrnsperger et al. 2000; Ehrnsperger et al. 1999). HspB1 is also deeply involved in the control of cytoskeletal organization (Dalle-Donne et al. 2001; Mounier and Arrigo 2002; Wettstein et al. 2012) and is well-known for its antiapoptotic and antioxidant properties (Arrigo 1998, 2001; Arrigo et al. 2005b; Bruey et al. 2000a; Mehlen et al. 1996b; Paul et al. 2002; Preville et al. 1999).

Interest in HspB1 has recently been boosted by important observations, such as its constitutive expression in different tissues, particularly in pathological conditions (Arrigo 2012; Franklin et al. 2005; Mymrikov et al. 2011), its ability to be secreted, and the discovery of several pathological mutations in its gene that induce neurodegenerative diseases (Benndorf et al. 2014; Boncoraglio et al. 2012). A direct consequence of HspB1 constitutive expression is an increased resistance of tissues to stress and injuries. The phenomenon is particularly intense in cancer cells where it stimulates their tumorigenic potential

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(Arrigo et al. 2015; Arrigo et al. 2007; Ciocca et al. 2015; Ciocca et al. 2013). Moreover, constitutively expressed HspB1 has recently been discovered to have a large number of apparently unrelated cellular functions that could result, as we recently proposed, of its ability to interact with many different protein partners (Arrigo 2013; Arrigo et al. 2015; Arrigo and Gibert 2012, 2013, 2014; Gibert et al. 2012a). Hence, increasing our knowledge of HspB1 structural organization in living cells could help in the understanding of how this protein can chaperone and modulate the activity and/or half-life of so many crucial client polypeptides (Arrigo 2013; Arrigo et al. 2015; Arrigo and Gibert 2013). The aim of this review is therefore to analyze and discuss the highly dynamic and complex patterns of HspB1 native size and phosphorylation in cells that undergo changes in their physiology or are exposed to different environmental conditions.

HspB1 phosphorylation

In the early days, HspB1 was known as a phosphoprotein whose degree of phosphorylation could be modified by agents such as phorbol ester, calcium ionophore, or stress conditions such as heat shock (Welch 1985). Human HspB1 contains three phosphorylatable serine sites (ser15, ser78, and ser82) located in its N-terminal part. Murine HspB1, also called Hsp25, has only two phosphorylated serine sites (ser15 and ser86). These phosphorylations result of the activity of several kinases (Kostenko and Moens 2009), particularly mitogen-activated protein kinases associated protein kinases (MAPK/MK2,3) which are themselves activated by phosphorylation by MAP p38 protein kinase. Other kinases that can phosphorylate human and murine HspB1 include MK5-PRAK, PKC γ , and PKD. Moreover, threonine 143 (Thr143) of human HspB1 appears phosphorylated by cGMP-dependent protein kinase. Consequently, HspB1 often displays complex patterns of phosphorylation since several transduction pathways, including those induced by stress, mitogen, and differentiation, activate these kinases.

HspB1 oligomerization

Small Hsps share, consequently of their crystallin homology domain, complex structural organization that allow the formation of dynamic homo-oligomeric structures that display heterogeneous native molecular masses (from about 50 to 800 kDa, depending on the small Hsps). The phenomenon has been extensively studied in the case of Human HspB1 and murine Hsp25. These studies were performed *in vitro* with the purified proteins (Ehlersperger et al. 1999) or by rapidly analyzing their native size after cell lysis (Arrigo 2007, 2011; Arrigo et al. 1988; Arrigo and Welch 1987; Basha et al. 2011; Garrido 2002; Lambert et al. 1999; Paul et al. 2010; Simon

et al. 2013). It has been proposed that the alpha-crystallin domain of HspB1 is responsible for its ability to form stable dimers that can further multimerize through intermolecular interactions mediated by the phosphorylation-sensitive N-terminal domain (Lambert et al. 1999). These dimers are dependent on the unique cysteine residue present in the alpha-crystallin domain of either murine (C141) or human (C137) HspB1 (Diaz-Latoud et al. 2005) and on the beta-7 strand that plays a pivotal role in the intersubunit contacts of several human small heat shock proteins (Mymrikov et al. 2010). Deletion of the unique cysteine blocks dimers formation, alters HspB1 ability to multimerize, and knocks out its chaperone activity and ability to interact with polypeptides (Diaz-Latoud et al. 2005). Concerning the molecular structure, X-ray analysis of HspB1 crystallin domain has been reported (Baranova et al. 2011; Rajagopal et al. 2015), but unfortunately not that of the entire molecule. Even if this X-ray analysis is obtained, HspB1 will still remain a challenging polypeptide because of its *in vivo* dynamic structural organization.

Changes in HspB1 native size: oligomerization or interaction with protein partners?

A major problem that researchers are facing when studying the *in vivo* native molecular size of HspB1 is to understand whether it depends of its level of oligomerization or of its interaction with protein partners having different molecular weights. Probably both phenomena occur, but this is a very difficult problem to solve since, in cells, the percentage of HspB1 polypeptides interacting with partners can be highly variable. For example, when I purified HspB1 for the first time, I took advantage of its ability to accumulate in large structures during heat shock recovery. A large fraction of HspB1 purified from these structures was in the form of high molecular weight oligomers (Arrigo and Welch 1987) and not in the form of small oligomers associated with stress-altered polypeptides. So, care should be taken when studying *in vivo* HspB1 native size.

Role of phosphorylation in regulating HspB1 native size

What is the role of HspB1 phosphorylation towards oligomerization or interaction with protein partners? Mutants have been used to test if its native size was directly linked (or not) to its degree of phosphorylation. From these studies, it is rather difficult to conclude since, in cells, modulation of HspB1 native size in absence of phosphorylation is well-known to occur. For example, it is generally accepted that an intense phosphorylation of the three serine sites present in the N-terminal domain induces the dissociation of HspB1 large

oligomers. A phenomenon that can be observed in tissue culture cells when phosphorylatable serine sites are replaced by phosphomimicry aspartic acids (Mehlen et al. 1997a; Rogalla et al. 1999; Vertii et al. 2006) or as mentioned below when cells are exposed to intense stress (heat shock, oxidative stress) (Kato et al. 1994; Preville et al. 1998b; Simon et al. 2013). However, caution should be taken since this conclusion does not hold true when cells are starved (Mehlen and Arrigo 1994) or when HspB1 is expressed in tissues (Bruey et al. 2000b). In that regard, we compared the organization of HspB1 wild type and mutants (serine residues replaced by alanine to mimic the non-phosphorylated protein or aspartate to mimic the phosphorylated protein) in cancer cells as well as in tumors formed by these cells. In growing cells (before being inoculated in the animal) and, in contrast to the alanine mutant, it was confirmed that the aspartate mutant could not form large structures. Of interest, the phenomenon was overcome by growing the cells *in vivo* (tumors formation) either in syngeneic animals or nude mice (Bruey et al. 2000b). Further studies dealing with cell confluency revealed that cell-cell contacts, such as those that exist in tissues and tumors, drastically increase HspB1 native size whatever the status of phosphorylatable serines could be (see also below). Whether this is due to HspB1 interaction with high molecular partners involved in cell to cell interaction or to the unique formation of HspB1 large oligomers is unknown.

Dynamic changes in HspB1 phosphorylation and native size

Compared to the different members of the family of mammalian small Hsps, an intriguing property of HspB1 concerns the intense and complex changes in its phosphorylation and native size in response to modulations of growth conditions or physiological alterations of the cellular environment. Of interest, these dynamic changes are reversible and specific (Arrigo 2000, 2007, 2011; Arrigo and Gibert 2012; Arrigo et al. 1988; Bruey et al. 2000b; Mehlen and Arrigo 1994; Mehlen et al. 1997a; Paul et al. 2010; Simon et al. 2013; Welch 1985). Moreover, they occur independently of whether HspB1 is stress-induced or constitutively expressed. We have therefore proposed that an intracellular sensor activity is associated to HspB1 that records changes in cellular environment (Paul et al. 2010). As discussed below, these dynamic changes may reprogram HspB1 ability to bind and modulate the folding/activity and/or half-life of appropriate client protein partners whose function will be to help the cell to better adapt to physiological changes or stress conditions.

Cell growth and cell arrest

To analyze HspB1 native size, we have used mammalian cells of cancerous origin which constitutively express high loads of

HspB1, such as HeLa and T47D cells (Arrigo et al. 1988; Mehlen et al. 1995a; Paul et al. 2010), so a putative formation of heterooligomeric complexes with other small Hsps could only be a minor event (Arrigo 2013; Mymrikov et al. 2012).

The native size of HspB1 in exponentially growing HeLa cells was analyzed by size-exclusion chromatography followed by western blot detection (Arrigo et al. 2005a). A typical pattern of HspB1 structural organization is presented in Fig. 1a. It is quite intriguing to note that in growing HeLa cells, HspB1 distributes in three sub-populations characterized by different native sizes (<150, 150–400, and >400 kDa) and phosphorylated serines. The presence of HspB1 in the less than 150-kDa fraction suggests that dimers or tetramers interacting or not with protein partners exist in living cells. On the phosphorylated side, it is interesting to note that in contrast to phosphorylated serine 78 which is present only in the medium-sized population (150–400 kDa), phosphoserine 15 is recovered only in the less than 150-kDa fraction together with about 60% of the total HspB1 content of phosphoserine 82. The remaining of phosphoserine 82 (40%) is in the >400-kDa population (Fig. 1a, (a)). A schematic cartoon is presented to summarize the data (Fig. 1a, (b)).

Using the same approach, the effect of serum starvation and refeeding was tested since it was reported that serum stimulated HspB1 phosphorylation in quiescent rat embryo fibroblasts (Welch 1985). HeLa cells were therefore incubated 48 h in a medium containing 0.1% fetal calf serum before being analyzed. A drastic dephosphorylation of HspB1 is then observed concomitantly with a decrease in its native size (Fig. 1b) and DNA synthesis (Mehlen and Arrigo 1994). Following the refeeding of starved cells, DNA synthesis is upregulated, HspB1 phosphorylation is intense and large native sizes are reappearing (Fig. 1c) (Mehlen and Arrigo 1994).

In the analysis described above, cells were lysed in a buffer containing a non-ionic detergent which solubilizes all the cellular content of HspB1. In contrast, in the absence of detergent about half of HspB1 present in growing cells remains associated with particulate fractions (Mehlen and Arrigo 1994; Mehlen et al. 1995a; Paul et al. 2010). In growth-arrested cells, HspB1 is essentially a soluble protein (Mehlen and Arrigo 1994) while upon refeeding with serum for 60 min, a fraction (about 30%) of total HspB1 in the form of small unphosphorylated structures redistributes in the particulate fractions. Only the remaining fraction of HspB1 present in the soluble fraction displays an enhanced phosphorylation and accumulates in large structures. During the recovery period after serum starvation, more than 48 h are then necessary to recover a cellular distribution, native size, and phosphorylation of HspB1 similar to that observed in untreated growing cells. Concerning the particular association of HspB1 with detergent sensitive structures of growing cells, this is probably an F-actin integrity-dependent phenomenon since it is not observed in cytochalasin D-treated cells (see below). HspB1 probably associates with the plasma membrane cytoskeleton compartment

Fig. 1 Native molecular size of HspB1 in growing, starved, and refed cells. **a** Exponentially growing HeLa cells. Cells were lysed in a buffer containing 0.1% Triton X-100 and the 20,000-g supernatant was loaded on a gel filtration column (size-exclusion chromatography) equilibrated and developed in lysis buffer devoid of detergent as already described (Arrigo et al. 2005a). Immunoblot analysis of the proteins present in pooled fractions of different molecular native masses (<150, 150–400, and >400 kDa) was performed using antibodies that are specific to either total or phosphorylated (Ser15-P, Ser78-P, or Ser82-P) HspB1. (a) Immunoblot analysis. (b) Immunoblot quantification and representative scheme. The percentage of total HspB1 present in each oligomeric size fraction is presented as well as a schematic representation of HspB1

phosphorylation using color chart to distinguish between dephosphorylated (40 to 60% of total HspB1) and phosphorylated (serines 15, 78, and 82) HspB1 polypeptides. It is hypothesized that non-phosphorylated and phosphorylated HspB1 polypeptides have the same ability to oligomerize and form chimeric structures. Partially reprinted from Paul et al. Dynamic processes that reflect antiapoptotic strategies set up by HspB1 (Hsp27). *Experimental Cell Research* 316, 1535–1552 (2010) with permission from Elsevier. **b** Same as **a** (b) but in this case, HeLa cells were starved for 48 h in a medium containing 0.1% serum before being analyzed. **c** Same as **b** but starved cells were refed for 60 min in a medium containing 15% serum

where actin nucleation occurs (Pichon et al. 2004; Tsvetkova et al. 2002), a phenomenon that obviously does not occur in growth-arrested cells. Moreover, another important factor that modulate HspB1 structural organization is cell confluency. Indeed, when cells reach confluence, they stop growing and consequently several properties of HspB1 are modified. First, the level of HspB1 is often upregulated. Second, the three size populations of HspB1 are lost, and its native size drastically increases as well as its phosphorylation and ability to protect against drugs (Bruey et al. 2000b; Garrido et al. 1997). Moreover, this point also clearly illustrates that the behavior of HspB1 can be different whether it is expressed in isolated cells or in tissues.

Cell differentiation

Differentiation programs are often associated with a transient increase in HspB1 expression (Arrigo 2005). The

phenomenon occurs during the early phase of the differentiation process concomitantly with the gradual growth arrest that precedes terminal differentiation. Amongst the differentiating cells expressing HspB1, one can cite Erlich ascite cells (Gaestel et al. 1989), embryonal carcinoma and stem cells (Mehlen et al. 1997b; Stahl et al. 1992), normal B and B lymphoma cells (Spector et al. 1992), osteoblasts (Shakoori et al. 1992), promyelocytic leukemia cells (Chaufour et al. 1996, Spector et al. 1995; Spector et al. 1994; Spector et al. 1993), normal T cells (Hanash et al. 1993), keratinocytes (Arrigo and Ducasse 2002; Duverger et al. 2004; Jantschitsch et al. 1998; Kindas-Mugge and Trautinger 1994), endometrium cells (Devaja et al. 1997), developing neuronal cells (Loones et al. 2000; Mehlen et al. 1999), chondrocytes (Favet et al. 2001), cardiomyocytes (Davidson and Morange 2000), muscle cells (Ito et al. 2001), and erythroid cells (de Thonel et al. 2010). A major finding was that impairment of HspB1 expression aborted differentiation and

triggered apoptosis, as shown in promyelocytic cells (Chaufour et al. 1996), embryonic stem cells (Mehlen et al. 1997b), cardiomyocytes (Davidson and Morange 2000), olfactory neurons (Mehlen et al. 1999), and erythroid cells (de Thonel et al. 2010). A similar role of HspB5 was reported during myogenic differentiation (Kamradt et al. 2002). Unfortunately, it is still not known why these proteins are essential to avoid abortion of the differentiation processes. Hypothesis could be that, through their chaperone activity, they protect cells undergoing drastic changes in their protein and cytoskeletal organization, phenomena that could raise junk polypeptides and trigger apoptosis (Arrigo 2005).

Hence, as mentioned above, knowledge of the structural organization of HspB1 appears as a mandatory requirement to unravel its function in cells committed to differentiate. Unfortunately, only few studies have analyzed the oligomerization and phosphorylation of HspB1 during differentiation processes and those studies were performed several years ago before antibodies specific to phosphorylated HspB1 were available. Analysis of these “old” data nevertheless reveals that the accumulation of HspB1 is associated with drastic dynamic changes in its phosphorylation and native size. For example, shortly after the induction of keratinocyte differentiation, a transient hyperphosphorylation of HspB1 occurs which is essential for the expression of differentiation markers. Later, the chaperone-active form of HspB1 plays a major role in the dynamics of keratin filament networks (Duverger et al. 2004). Similarly, in retinoic acid-mediated granulocyte differentiation of human leukemic cells, HspB1 phosphorylation transiently increases before cellular growth is altered. This early phenomenon is followed by a drastic increase in HspB1 native size that occurs concomitantly with the maximal accumulation of this protein (Chaufour et al. 1996). A rather identical picture is observed during the pluripotential differentiation of embryonic murine CGR8 stem cells triggered by leukemia inhibitory factor (LIF) withdrawal (Mehlen et al. 1997b). In growing non-differentiated CGR8 cells and during the first hour of the differentiation process HspB1 is mainly observed in the form of phosphorylated small structures, but it accumulates thereafter as an unphosphorylated polypeptide. Twenty-four hours later, HspB1 is then in the form of medium- and large-sized structures. The shift towards large native sizes is no more observed after 48 h and HspB1 is back in the form of small structures but, in contrast to those observed in undifferentiated growing cells, they are unphosphorylated.

Cells exposed to apoptotic inducers

Back in 1996, we reported that HspB1 drastically decreases the death efficiency of apoptotic inducers (Arrigo 2000 ; Arrigo et al. 2007; Bruey et al. 2000a; Concannon et al.

2003; Garrido et al. 1999; Mehlen et al. 1996b; Paul et al. 2002; Samali and Cotter 1996). Unlike stresses that misfold proteins, apoptotic programs per se do not induce HspB1 synthesis. Hence, in cells or tissues where it is constitutively expressed, such as in many cancer cells, a physiological antiapoptotic role is played by HspB1 (Gibert et al. 2012b; Hayashi et al. 2012). HspB1 acts both upstream and downstream of mitochondria. The upstream effect occurs at the level of different signaling pathways such as those triggered by alterations in F-actin or nucleus architecture integrity (Paul et al. 2002; Samali et al. 2001) or those that are Bax, Bid, and Akt dependent (Havasi et al. 2008; Paul et al. 2010; Rane et al. 2003; Wu et al. 2007). Downstream of mitochondria, by interacting with cytochrome c once it is released from mitochondria (Bruey et al. 2000a; Paul et al. 2002), HspB1 impairs pro-caspase-9 activation (Garrido et al. 1999). Moreover, by interacting with pro-caspase-3, it attenuates the activation of this highly efficient effector caspase by the apoptosome-associated caspase-9 (Pandey et al. 2000). The downstream effects of HspB1 were further confirmed *in vitro* by showing that recombinant HspB1, which is in the form of large unphosphorylated oligomers, added to cytosolic extracts decreases cytochrome c-mediated caspases activation (Bruey et al. 2000b). Less information is available concerning the extrinsic pathway, in spite of the fact that HspB1 small oligomers can interact with DAXX and block the apoptotic pathway downstream of this protein (Charette et al. 2000). Studies using mutants expressed in tissue culture cells revealed that non-phosphorylatable HspB1 retains antiapoptotic activity while the phosphomimicry mutant which only forms small structures had no antiapoptotic activity (Bruey et al. 2000b). In contrast, and as already mentioned above, in tumors, this mutant regained its ability to increase its native size, a phenomenon associated with a renewed antiapoptotic activity. Hence, the antiapoptotic activity of HspB1 appears to require the formation of large structures (Bruey et al. 2000b).

To gain more information towards the structural organization of HspB1 in cells undergoing apoptosis, we analyzed the behavior of this Hsp in HeLa cells exposed to stimuli known to induce apoptosis through different pathways: such as those triggered by Fas agonist antibody (Fas Ab), etoposide, staurosporine, and cytochalasin D (Paul et al. 2010). We first reported that the intracellular localization of HspB1 during early apoptosis (before nucleus fragmentation) is inducer specific. In etoposide and Fas Ab-treated cells, in spite of some diffuse cytoplasmic distribution, HspB1 localizes together with F-actin in cell surface blebs (Paul et al. 2010), known as plasma membrane cytoskeleton compartments (Pichon et al. 2004). The phenomenon is not observed in response to staurosporine and cytochalasin D treatments that rapidly destroy F-actin and do not induce membrane blebbing (Huot et al. 1998). The different HspB1 size fractions described in Fig. 1a were then analyzed in cells exposed to the different

apoptosis inducers. As seen in Fig. 2a, in cells exposed to Fas Ab, HspB1 has the gradual tendency to concentrate in large structures (>400 kDa). However, it is quite intriguing to note that the distribution of phosphorylated HspB1 polypeptides remains close to that observed in untreated cells (Fig. 1a). In cells exposed to etoposide (Fig. 2b), HspB1 has also the tendency to concentrate in >400 kDa structures but the distribution of phosphorylated HspB1 differs from that observed in untreated and Fas Ab-treated cells. In this case, phosphorylated serine 15 (P-ser 15) is recovered in the three size populations while P-ser78 redistributes in the small and large structures. Of interest, after 12 h in the presence of these drugs, the size distribution and phosphorylation of HspB1 are back to normal. When a similar experiment is performed with cells exposed to either staurosporine (Fig. 2c, d) or cytochalasin D (Fig. 2e, f), a different response is observed since these inducers rapidly redistribute HspB1 in small structures (<150 kDa) before inducing the reformation of large ones (>400 kDa). As in the case of etoposide, the distribution of phosphorylated HspB1 polypeptides is deeply altered. Analysis after 1 and 3 h of staurosporine reveals that these changes are dynamic. Indeed, after 1 h of treatment, the remaining large structures contain only a small percentage of P-ser82 and the medium-sized ones (150–400 kDa) are enriched in P-ser15. In addition, P-ser78 is recovered in the <150-kDa fraction. After 3 h of treatment, P-ser78 and P-ser82 are now abundant in the >400-kDa fraction. Analysis

of the events occurring in cytochalasin D-treated cells reveals another distribution of HspB1-phosphorylated polypeptides. As in the early event mediated by staurosporine, the large structures (>400 kDa) are dephosphorylated, a phenomenon still observed after 3 h of treatment with cytochalasin D. The major changes are now at the level of the medium-sized structures that contain the three phosphorylated polypeptides of HspB1. As in the case of Fas and etoposide, after 12 h of treatment the native size profile of HspB1 is back to normal. Hence, two types of inducers could be defined—etoposide and Fas Ab—which shift HspB1 towards large structures and - staurosporine and cytochalasin D - which rapidly concentrate HspB1 in small ones. In that regard, it is intriguing to note that the rapid redistribution of HspB1 in the <150-kDa population occurs in cells exposed to drugs that induce a rapid disruption of F-actin architecture. In these conditions, HspB1 may act towards the plasma membrane cytoskeleton compartment (Paul et al. 2010) before taking care of the apoptotic machinery where the >400-kDa population is efficient (Bruey et al. 2000b). However, the precise role played by each phosphoserine site in these processes is still not known (Paul et al. 2010). It is also interesting to note that in response to the different apoptotic inducers, HspB1 phosphorylation increases until about half of the cellular content of the polypeptide is phosphorylated. The phenomenon is transient and has about the same time duration (about 12 h) as the dynamic redistribution of HspB1 native sizes. Indeed, the intriguing

Fig. 2 HspB1 native molecular size in cells exposed to apoptotic inducers. HeLa cells were treated with apoptotic inducers for different time periods as previously described (Paul et al. 2010): **a** 20 ng/ml Fas agonist antibody + 5 ng/ml actinomycin D for 6 h, **b** etoposide 500 μ M for 3 h, **c–d** 0.125 μ M staurosporine for 1 and 3 h, and **e–f** 0.5 μ M cytochalasin D for 3 and 6 h. Cells were processed and immunoblot

analysis of the proteins present in the pooled fractions were performed as described in Fig. 1. The percentage of total HspB1 present in each oligomeric size fraction and a schematic representation of HspB1 phosphorylation using color to distinguish between dephosphorylated and phosphorylated HspB1 polypeptides are presented

aspect of HspB1 structural changes is their transient nature. They begin to be detectable early when DEVDase activation is weak. Then, their maximal intensity correlates with the logarithmic activation phase of DEVDase. Thereafter, HspB1 structural organization is back to normal while the activity of caspases is still rising. Based on the fact that, in the absence of HspB1, DEVDase caspase activity is triggered more rapidly and is 2 to 3 times more intense (Paul et al. 2010), it can be concluded that the transient inducer-specific changes in HspB1 structural organization are aimed at decreasing the inducers effectiveness to activate caspases. HspB1 appears therefore to have multiple and complex strategies to negatively modulate differentially induced apoptotic programs.

Stressed cells

Heat shock

HspB1 is well-known to protect cells against heat shock treatments (Landry et al. 1989). In addition to its upregulated synthesis in response to heat shock, HspB1 undergoes drastic changes in its localization, native size and phosphorylation (Arrigo et al. 1988). One important parameter is the intensity of the stress since drastic ones can induce the protein to migrate into the collapsing nucleus (Arrigo et al. 1988). Analysis of the effects induced by sub-lethal heat shock challenges revealed that HspB1 first accumulates as small structures

(Arrigo et al. 1988; Kato et al. 1994) (Fig. 3a) and subsequently in large ones (Arrigo et al. 1988) (Fig. 3b), a property used for its purification (Arrigo and Welch 1987). Another important point concerns the reversibility of the phenomenon during heat shock recovery and its absence in thermotolerant cells (Arrigo et al. 1988). It has then been proposed that an early event induced by heat shock, due to the intense phosphorylation of HspB1, is the dissociation of its large oligomeric structures (Kato et al. 1994). Our analysis revealed that the phenomenon is a bit more complex since in heat shock treated cells the remaining low level of large structures (>400 kDa) are not phosphorylated and therefore devoid of P-ser82 (Fig. 3a). Moreover, the medium-sized structures (150–400 kDa) now contain the three phosphorylated serines instead of only P-ser78 as in growing cells. Hence, the heat-induced changes are not only characterized by a dissociation of HspB1 oligomeric structures but also by a size redistribution of some of its phosphorylated structures. A second important point concerns the >400-kDa population that reforms during heat shock recovery which is phosphorylated differently than that observed in growing cells (Paul et al. 2010). As illustrated in Fig. 3b, these >400 kDa structures are specifically phosphorylated at the level of serines 15 and 82 while those in growing cells only contain P-ser82. Medium-sized (150–400 kDa) ones are also differently phosphorylated since they contain P-ser15. These intriguing observations suggest that specific HspB1 structures accumulate in heat shock-treated cells. They differ from those observed in growing cells and in cells exposed to the different conditions that have been

Fig. 3 HspB1 native molecular size in cells exposed to stress. As in Fig. 2 except that cells were exposed to stress conditions. Growing HeLa cells were exposed to a heat shock challenge of 1 h at 42 °C (a) followed by a recovery period of 3 h at 37 °C (b). HeLa cells were also exposed to oxidative conditions generated by 60 μ M of menadione for 2 h (c). Reducing stress was induced by incubating HeLa cells with 30 mM *N*-acetylcysteine for 16 h (d). Similar effect of reducing stress was observed in T47D cells expressing the seleno-dependent enzyme glutathione peroxidase (Mehlen et al. 1995a)

described here. What could this mean? One hypothesis could be that HspB1-active structures in heat-stressed cells are special ones related to the “holdase molecular sponges activity” described *in vitro* to entrap stress-denatured client polypeptides before ATP-dependent molecular chaperones (Hsp70, Hsp90, and co-chaperones) could take care of them (Ehrnsperger et al. 2000; Ehrnsperger et al. 1997; Ehrnsperger et al. 1999; Lee et al. 1997). However, as mentioned before, after heat shock HspB1 also forms large structures because of its stimulated oligomerization (Arrigo and Welch 1987). Hence, the >400-kDa fraction probably contains two types of populations of specifically phosphorylated oligomers: small- and medium-sized ones entrapping denatured client polypeptides and isolated large ones.

Oxidative stress

Another intriguing property of HspB1 concerns its antioxidant property that generates a cellular protection against oxidative stress inducers and pathological conditions (Arrigo 1998, 2001; Mehlen et al. 1995a, b, c; Merendino et al. 2002; Simon et al. 2013; Wytenbach et al. 2002). HspB1 expression, which is not upregulated by oxidative stress, decreases intracellular reactive oxygen species (ROS) and nitric oxide levels. It also upholds glutathione in its reducing form and mitochondrial membrane potential ($\Delta\Phi_m$) (Arrigo 2007; Mehlen et al. 1996a; Preville et al. 1999; Rogalla et al. 1999). In addition, it consolidates intracellular redox homeostasis by decreasing iron intracellular levels (Arrigo et al. 2005b; Chen et al. 2006) and consequently hydrogen radical production by the Fenton reaction. We and others reported that HspB1 stimulates the activity of glucose 6-phosphate dehydrogenase (G6PD) (Preville et al. 1999; Yan et al. 2002) through an interaction of this reducing enzyme with its small and heavily phosphorylated oligomers (Arrigo 2013; Cosentino et al. 2011). Hence, the presence of HspB1 strongly attenuates protein oxidation, DNA damages, lipid peroxidation, and cytoskeleton architecture disruption (Mehlen et al. 1996a; Preville et al. 1998a; Preville et al. 1999).

In response to oxidative conditions, a rapid disappearance of HspB1 large (>400 kDa)- and medium (150–400 kDa)-sized structural populations and an intense phosphorylation of the three phosphorylated sites are detected (Fig. 3c), see also (Simon et al. 2013). This favors the interaction with G6PD and may trigger other still unknown effects such as the elimination of oxidized polypeptides that cannot be detoxicated and refolded. In contrast to heat shock, treating cells with oxidative agents is not reversible, so it is not possible to study a late structural behavior of HspB1. To try to overcome this problem cells were exposed to the inflammatory cytokine TNF α which stimulates HspB1 phosphorylation (Arrigo 1990) and generates, amongst many other events, a discrete oxidative stress attenuated by the expression of

HspB1 (Mehlen et al. 1995c, 1996a). In TNF α treated cells, HspB1 structural behavior is different from that induced by oxidative agents (Mehlen et al. 1995b). Indeed, during the first 2 h of treatment, concomitantly with a burst of ROS, HspB1 rapidly accumulates in large unphosphorylated structures (>400 kDa). Then, by 4 h, its native size drastically decreases (<200 kDa). During this phenomenon, phosphorylated HspB1 is mostly recovered in the small- and medium-sized structures. Analysis of the effect mediated by unphosphorylatable mutant (ser to ala) or P38 MAP kinase inhibitor clearly pointed to the fact that the large unphosphorylated structures (>400 kDa) that form early during TNF α treatment are essential for the protective activity of HspB1 (Mehlen et al. 1997a; Preville et al. 1998b). Whether these structures result of HspB1 small oligomers interacting with large polypeptides or to a higher level of HspB1 oligomerization or even to both types of events is not known.

We next analyzed whether in growing cells the fate of the intracellular redox state could modulate HspB1 structural organization. In that respect, it was observed that the over-expression of the antioxidant seleno-glutathione peroxidase (GSHPx) redistributes HspB1 in detergent sensitive particulate fractions and induces its dephosphorylation but does not alter its size pattern (Fig. 3d) (Mehlen et al. 1995a). In these antioxidant conditions, no change in HspB1 native size and phosphorylation could be triggered by TNF α . In contrast, heat shock rapidly induces HspB1 phosphorylation. This suggests that the signal transduction mechanisms leading to HspB1 phosphorylation in response to heat shock does not depend on ROS while that observed in growing cells (MAP kinase activating P38) is clearly redox state dependent (Mehlen et al. 1995a). Another surprising observation was that the modulation of reduced glutathione level, induced by treating cells with either glutathione monoester (300% increased level of glutathione) or buthionine sulfoximine (BSO, 99% depletion of glutathione) drastically alters the pattern of HspB1 native size. A drastic increase in glutathione shifts HspB1 towards the >400 kDa form while its depletion induces the accumulation of HspB1 in dimers. Hence, HspB1 structural organization appears highly sensitive to intracellular redox status.

HspB1 in pathologies

Cancer

High levels of Hsps and HSF1 (heat shock transcription factor) are usually expressed in cancer cells consequently of the accumulation of mutant proteins that alter protein homeostasis (Calderwood 2012; Calderwood et al. 2006). This addiction to chaperones allows cancer cells to be more aggressive and resistant (Arrigo 2012; Ciocca et al. 2013). Concerning

HspB1, its expression correlates with a poor clinical outcome in many types of cancers. It is essential for protecting cancer cells against apoptosis or other types of death triggered by the immune system (Aloy et al. 2008; Hayashi et al. 2012; Mehlen et al. 1996b; Paul et al. 2010; Sun et al. 2015). HspB1 is also required for cancer cells growth, tumorigenic activity (Bruey et al. 2000b; Garrido et al. 2006; Garrido et al. 1998), metastasis formation and dissemination (Bausero et al. 2006; Fanelli et al. 2008; Gibert et al. 2012b; Lemieux et al. 1997; Nagaraja et al. 2012), maintenance of cancer stem cells (Wei et al. 2011), and modulation of the epithelial to mesenchymal transition process (Choi et al. 2016; Wettstein et al. 2013). It also acts in survival pathways (Rane et al. 2003; Schultz et al. 2012) and counteracts host anticancer response, such as senescence (O’Callaghan-Sunol et al. 2007). HspB1 ability to provide cancer cells with resistance to many anticancer drugs, which in turn, unfortunately, stimulates HspB1 expression has also been described a long time ago (Huot et al. 1991). HspB1 is therefore considered as a highly interesting therapeutic target (Acunzo et al. 2014; Arrigo et al. 2015; Arrigo and Gibert 2014; Fossa and Cichero 2015; Katsogiannou et al. 2014).

Despite the large spectrum of publications related to HspB1 in cancer cells, few informations are available concerning its structural organization when it is expressed in these pathological cells. Some reports are dealing with HspB1 phosphorylated status which could play a key role in gemcitabine-induced apoptosis of pancreatic cancer cells (Nakashima et al. 2011) or be associated with cancer development and progression (Katsogiannou et al. 2014). Hence, a major study linked to this important question remains the one we published in 2000 in collaboration with Carmen Garrido’s team in Dijon (Bruey et al. 2000b) using rat colon cancer REG cells that yield immunogenic and regressive tumors when injected in syngeneic animals but aggressive ones when they are genetically manipulated to express human HspB1 (Garrido et al. 1998). REG cells were therefore transfected with either wild-type or mutated human HspB1. One mutant was the non-phosphorylatable HspB1 (in which the phosphorylatable serine residues have been replaced by alanine to mimic the non-phosphorylated protein) and the other one was the phosphomimicry HspB1 (in which the phosphorylatable serine residues have been replaced by aspartate to mimic the negative charge of the phosphorylated protein). In growing REG-transfected cells, wild-type HspB1 formed small-sized, medium-sized, and large structures while the alanine mutant was mainly in the form of the large ones. Both proteins demonstrated antiapoptotic activity confirming the important role played by the large HspB1 structures. In contrast, HspB1 aspartate mutant had a small native size and no antiapoptotic activity. In vivo, the subcutaneous injection of REG cells expressing wild-type HspB1 yielded large tumors. Of great interest was the finding that both cells

expressing either the alanine or the aspartate mutant, known to display different antiapoptotic activities when cultured in vitro, had an unexpectedly tumorigenic effect similar to that of wild-type HspB1. Analysis of the structural organization revealed that the aspartate mutant of HspB1 was now able to form large structures and to have antiapoptotic activity. This intriguing property of the aspartate mutant was lost when the analysis was performed in cells obtained by dissociation of the tumors grown in vivo and then cultured for 15 days in vitro. Hence, as mentioned before, the overcoming effect was a consequence of the elevated cell density inside tumors. So, whatever the status of phosphorylatable serines is, cell-cell contacts induce the formation of HspB1 large structures, a phenomenon probably linked to the fate of the cytoskeleton. This study is another nice example of the crucial role of the cellular environment on HspB1 structural organization. It also points to the fact that observations made in tissue cultured cells may not exactly represent what is going on in vivo.

Neuropathologies

Elevated levels of HspB1 are observed in cells expressing proteins prone to aggregate or fibrillate and therefore can interfere with pathological processes leading to neurodegenerative diseases (Firdaus et al. 2006; Outeiro et al. 2006; Perrin et al. 2007; Wyttenbach et al. 2002). Moreover, pathological mutations that inhibit the chaperone activity of HspB1 provoke pathological diseases, such as amyotrophic lateral sclerosis (ALS), axonal Charcot-Marie-Tooth disease, and inherited peripheral and motor neuropathies (Benndorf et al. 2014). However, the structural organization of HspB1 that is active in these pathological cells is unfortunately still unknown. Mutations probably alter the ability of HspB1 to interact and chaperone crucial polypeptides counteracting the development of these degenerative diseases.

Conclusions

As shown here, the environment of the cell and its physiology have a huge impact on HspB1 native size and phosphorylation. In that regard, it is interesting to distinguish between the effects mediated by drastic stress and the modulations of the structural organization of constitutively expressed HspB1 in cells exposed to environmental changes or undergoing changes in their growth or differentiation program. In the latter cases, specifically phosphorylated HspB1 oligomers of particular size are probably dedicated to interact with particular clients in a defined cellular condition. In all the conditions that we and others have studied, the heat shock-specific behavior of HspB1 was never observed. This means that the changes in the phosphorylation, oligomerization, and native size of HspB1 are highly condition-specific. Future investigations

will have to determine what are the respective roles of oligomerization and interaction with protein partners in the modulation of HspB1 native size. Another, very important point raised by these studies is that observations made in cultured cells may not exactly represent what is going on in vivo. Since HspB1 is a protein chaperone that can bind polypeptides and modulate their folding, this suggests that HspB1 is a molecular sensor that responds to modifications of the cellular physiology by changing its structural organization in order to create interacting platforms for specific client polypeptides (Arrigo 2013; Arrigo et al. 2015; Arrigo and Gibert 2012; Katsogiannou et al. 2014). This hypothesis is supported by our recent observation that, in the same human cell, specific phospho-oligomeric structures of HspB1 can interact with different client proteins and modulate their half-life (Gibert et al. 2012a). Hence, a major unanswered question relates to the molecular signaling mechanisms that regulate the structural dynamic of HspB1 required to recognize and interact with diverse client repertoires in defined cellular conditions. Moreover, in vivo analysis of HspB1 platforms is a difficult task to be studied by conventional structural biology methods that will need new approaches. On the positive side, we already know that in vitro the quaternary structure of small Hsps has a high level of dynamic plasticity (Basha et al. 2011; Stengel et al. 2010) modulated by phosphorylation (Hayes et al. 2009). Hence, through its protein interactome (Arrigo 2013; Arrigo et al. 2015), HspB1 has immense cellular implications in the normal life of the cell and appears therefore to act similarly as Hsp90 (McClellan et al. 2007; Neckers 2002; Taipale et al. 2010).

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