Chapter 7
Pathways of Hsp70 Release: Lessons from Cytokine Secretion

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Abstract Heat shock proteins (HSP) have an essential role in the cytoplasm of all cells in mediating protein quality control. However, in addition to these molecular chaperone properties, HSP play additional extracellular roles as mediators of inflammation and immunity. Because of their lack of a signal sequence and exclusion from the classical secretion pathways, it was initially assumed that extracellular HSPs resulted from cell necrosis and release of cell contents. However, non-canonical protein secretion pathways have been described, and have been studied intensively for the cytokine interleukin-1β (IL-1β). At least four mechanisms have been described, including: (1) IL-1β entry into secretary lysosomes and secretion into the medium along with lysosomal enzymes; (2) shedding of microvesicles from the membrane that contain IL-1β; (3) release of IL-1β directly through the membrane bound to a conjectured secreted-protein-transporter and; (4) formation of multivesicular bodies containing IL-1β and MHC class II within recycling endosomes and release of IL-1β entrapped in exosomes enclosed within the vesicles. Each of these mechanisms may be operative in HSP secretion and there is particularly strong evidence for mechanisms (1) and (4), in which either free Hsp70 is released through the endolysosome pathway or HSPs are secreted in association with exosomes. HSPs released through these mechanisms have intercellular signalling properties and can regulate phagocytosis, T and NK cell activation and release of cytokines in the acute inflammatory response. We discuss triggering mechanisms for HSP release, the pathways involved in HSP traverse of lipid membranes and the physiological consequences of HSP secretion.

7.1 Introduction

Heat shock proteins (HSP) are essential intracellular chaperones required to maintain the cellular proteome in a functional and folded conformation (Calderwood et al. 2009). However, it has slowly become apparent that HSPs can also be found in the extracellular spaces and in the circulation (Hightower and Guidon 1989; Pockley

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2002; Pockley et al. 1998; Tytell et al. 1986). The subject of extracellular chaperones is also discussed in Chaps. 3 and 18. The existence of such 'displaced' HSP molecules might be regarded as evidence of: (i) cell necrosis and release from necrotic bodies; (ii) active secretion from viable cells, or; (iii) a consequence of both processes (Mambula and Calderwood 2006a, b; Mambula et al. 2007). It seems unlikely that most extracellular HSPs could function significantly as molecular chaperones when secreted, due to their requirement for ATP and co-chaperones, and indeed an additional class of extracellular chaperones exists in the form of plasma proteins such as clusterin (Wilson et al. 2008). However, extracellular HSP have been shown to possess potent immunobiological properties and can induce both immune stimulation and immune suppression (Srivastava 2005; Srivastava and Maki 1991; van Eden et al. 2005; Wieten et al. 2009). Therefore insights into HSP release mechanisms would seem potentially valuable in unravelling their immune functions. In addition, the mechanisms and processes involved in HSP release are not uniformly agreed on and further inspection of the published data may illuminate some of the controversial issues (De Maio 2010).

Most proteins targeted for release from cells are secreted by the canonical pathway, in which they are inserted co-translationally into the endoplasmic reticulum, progress through the Golgi apparatus and are then released into extracellular spaces (Halban and Irminger 1994; Stanley and Lacy 2010). Proteins released through this pathway encode a hydrophobic N-terminal signal sequence that is inserted into the ER membrane during translation and then is cleaved before secretion (Halban and Irminger 1994; Stanley and Lacy 2010). However, not all secretion pathways employ this route and non-canonical routes exist for release of proteins devoid of signal sequences (such as HSPs) to be secreted. These non-classical pathways have been carefully explored in study of cytokines such as interleukin-1α (IL-1α), interleukin-1β (IL-1β) fibroblast growth factor (FGF), interleukin-15 (IL-15) and interleukin-18 (IL-18) and the mechanisms deduced may serve as useful paradigms for understanding HSP secretion (Andrei et al. 1999; Duitman et al. 2008, 2010; MacKenzie et al. 2001; Prudovsky et al. 2003).

7.2 HSP Secretion: Lessons from Cytokines

IL-1β is an essential cytokine that mediates the acute phase response and fever and is rapidly synthesized on free ribosomes in response to inflammatory signals in the cytoplasm, prior to secretion (Dinarello 2009, 2010). Secretion of the leaderless IL-1β polypeptide in monocytes, macrophages and dendritic cells appears to require at least two triggering signals at the cell surface, including: (1) transcriptional activation by agents such as bacterial endotoxins and; (2) stimulation of release by extracellular ATP (Wewers 2004). There are at least four known pathways for IL-1β release that are each supported by convincing data and illustrated in Fig. 7.1. These include:

1. IL-1β entry into secretary lysosomes and secretion into the medium along with lysosomal enzymes and markers (Andrei et al. 1999, 2004).

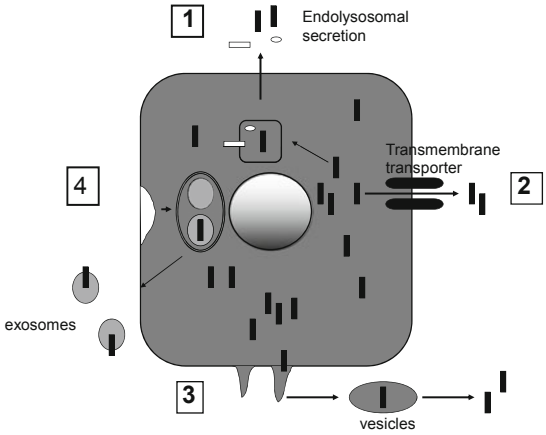

Fig. 7.1 Four pathways of non-canonical protein secretion. Proteins such as HSP (■) or IL-1β could be secreted through: (*1*) The endolysosomal pathway. HSP enter the lysosome after stress or other signals. Lysosomes also contain membrane LAMP1 (□) and intralysosomal cathepsin D (◯). When the secretory lysosome fuses with the plasma membrane, free HSP is released along with LAMP1 and cathepsin D. (*2*) Secreted HSP could also be transported across the membrane directly by an, as-yet-uncharacterized transporter as wit IL-1β. (*3*) The HSP may also enter membrane blebs formed after treatment with agents such as ATP. These membrane protuberances may be pinched off and released into the extracellular space as vesicles containing HSP. (*4*) HSP may become packaged in MVB. These are formed after endocytosis from the plasma membrane, when endosomes are formed and then vesicles (exosomes) subsequently bud from the interior of the endosome to form enclosed exosomes. Hsp70 has been found protruding from the exosomal surface. When MVB fuse with the plasma membrane, exosomes are released

2. Shedding of microvesicles from the membrane that form on stimulation with ATP and contain IL-1β (Pizzirani et al. 2007).
3. Release of IL-1β directly through the membrane bound to a conjectured secreted-protein-transporter that may, or may not be the ATP-binding-cassette-1 transporter ABC-A1 (Brough and Rothwell 2007).
4. Formation of multivesicular bodies containing IL-1β and MHC class II within recycling endosomes and release of IL-1β entrapped in exosomes enclosed within the vesicles (Qu et al. 2007).

In comparing secretion pathways of HSP with mechanisms related to IL-1β, it is as well to consider also differences between the two processes. IL-1β is expressed at minimal levels in unstimulated monocytes and macrophages and on stimulation synthesized as a larger polypeptide (pro-interleukin-1β) that must be processed by the enzyme caspase-1 before secretion (Ogura et al. 2006; Perregaux and Gabel 1994; Wewers 2004). Many tissue culture cells contain abundant levels of constitutively expressed Hsp70 and there is currently no evidence for a role of proteolytic processing in secretion.

7.3 Hsp70 Secretion via the Endolysosome Pathway

We have carefully examined the pathways of release of free Hsp70 from cells. As we did not know the nature of the proximal inducer of Hsp70 release, we initially used heat shock as a trigger for secretion, based on previous studies which indicated that mild heat shock can lead to non-canonical secretion of FGF and IL-1α (Prudovsky et al. 2003). We found that Hsp70 was secreted from two prostate cell lines, PC-3 and LnCap at both 40 °C and 43 °C (Mambula and Calderwood 2006b). Release occurred from viable cells only during the heat shock itself, ceased when cells recovered at 37 °C and appeared to be a direct response to the elevated temperature. Exposure to more toxic forms of thermal stress inhibited release, suggesting a secretion pathway with components sensitive to thermal denaturation (Mambula and Calderwood 2006a). On comparing the mechanisms of Hsp70 release to those utilized in secretion of IL-1β, we found that mechanisms 1 and 2, described above appeared to be operative in Hsp70 secretion (Mambula and Calderwood 2006b) (Fig. 7.1). Hsp70 release was reduced by the lysosomotropic agents: methylamine and NH_4Cl. In addition, Hsp70 became localized to the lysosomal fraction marked by cathepsin D during the heat shock and its release was accompanied by the trafficking of lysosomal surface marker, LAMP1, to the cell surface of heated cells (Mambula and Calderwood 2006b). A mechanism involving stress-mediated entry of Hsp70 into lysosomes, fusion of these organelles to the plasma membrane and release of contents to the extracellular milieu is indicated. Further studies also suggested that Hsp70 release may require ATP (as with IL-1β) and elevated levels of Mg^{++}, a known inhibitory condition for purinergic receptors inhibited secretion (Mambula and Calderwood 2006b). Results consistent with these findings were observed in human subjects *in vivo* in which elevated levels of HSP72 were strongly correlated with increases in external ATP (Ogawa et al. 2011).There was some support for secretion mechanism (3) in Hsp70 release in that inhibitors of the transporter protein ABC-B1 (glibemclemide and DIDS) inhibited thermally-induced Hsp70 secretion. It is possible that ABC-B1 could be involved in either entry of the Hsp70 into secretory lysosomes (1) or in direct secretion across the plasma membrane (3). In these studies we did not find evidence for Hsp70 in membrane-bounded microvesicles or vesicular bodies, as ultracentrifugation of cell medium failed to sediment detectable amounts of Hsp70. We have more recently examined these mechanisms in macrophage cell lines stimulated by microorganisms and PAMPs derived from *E. coli* (S.S. Mambula, A. Murshid & S.K. Calderwood, submitted). Stimulation by exposure to *E. coli* caused Hsp70 release from Raw 264.7 mouse macrophages coordinately with IL-1β and the lysosomal marker LAMP1. In addition, lysosomotropic agents inhibited bacterial induction of Hsp70 release suggesting a similar mechanism, involving the endolysosome pathway for chaperone release after *E. coli* exposure, as was seen with thermal stress. Free Hsp70 may be released through the endolysosomal pathway following exposure to microorganisms and can have a number of effects including bystander activation of phagocytosis (S.S. Mambula, A. Murshid & S.K. Calderwood, submitted) and cytokine induction in monocytes, macrophages and DC (Asea et al. 2000,

2002). The cell surface role of Hsp70 in recognising the Gram-negative component LPS is described by Triantafilou in Chap. 9.

7.4 HSP Release in Vesicles

A number of studies indicate that HSPs can be released when entrapped in lipid vesicles (mechanisms 2, 4) described above (Chen et al. 2006; Clayton et al. 2005; Elsner et al. 2007; Gupta and Knowlton 2007; Lancaster and Febbraio 2005; Mathew et al. 1995; Taylor et al. 2007; Thery et al. 1999; Xie et al. 2010). These studies indicate that such HSPs are present in exosomes derived from a wide array of cell types, including: reticulocytes, peripheral blood mononuclear cells (PBMCs), B cells, dendritic cells, hepatocytes, and a range of cancer cells (mesothelioma, colon cancer, K562, mammary carcinoma—reviewed in De Maio 2010 (see Fig. 7.1)). This process involves the internalization of regions of the plasma membrane into endosomes that recycle to the cytoplasm, with specific sorting of proteins in the endosomes into internal vesicles pinched off from the endosomal membrane, the exosomes (Chaput et al. 2006). These structures have been described as multivesicular bodies (MVB) and are involved in secretion of both IL-β and Hsp70 (Chalmin et al. 2010; Qu et al. 2007). MVB next fuse with the membrane leading to release of the exosomes into the extracellular spaces (Chalmin et al. 2010). The key question regarding exosomal release, as with each of the pathways of non-canonical secretion, is—how do proteins without signal sequences become inserted into/cross lipid membranes? Even more intriguingly, Hsp70 has been found in the exposed surface of the exosomes and may serve a recognition/signalling role in targeting the exosomes (Chalmin et al. 2010; Vega et al. 2008). These questions have been addressed in detail by A. De Maio and co-workers (Vega et al. 2008). These workers have shown heat shock-induced secretion of exosomal-like structures that they refer to as ECM (extracellular membrane), with Hsp70 exposed in the outer leaflet of the membrane. They have proposed a mechanism whereby Hsp70 can bind specifically to phosphatidylserine (PS) and then become an integral membrane protein associated with this lipid and promote ion channel forming activities (Arispe et al. 2004; Schilling et al. 2009). The mechanisms involved in this process are not clear although *flipase* molecules known to be present in the plasma membrane and that transport phosphatidylserined (PS) from the inner leaflet into the outer leaflet of the membrane could potentially be involved (Vega et al. 2008). Hsp70 could thus be transported as a passenger when Hsp70-bound PS molecules are flipped from the inner to the outer leaflet of the plasma membrane. An alternative mechanism for non-canonical secretion of FGF during heat shock was proposed by Prudovsky and coworkers and involves conversion of the secreted protein into a partially unfolded "molten globule" form that could then cross the membrane (Prudovsky et al. 2003). Exosomes have been shown to have a specialized lipid content compared to the bulk plasma membrane, with elevated cholesterol and sphingolipids characteristic of detergent resistant microdomains or lipid rafts (De Maio 2010 see also Chap. 6). Hsp70 has been detected previously within the lipid raft fraction (Triantafilou et al. 2002—see also Chap. 9). Exosomes were also

previously shown to be enriched in GPI-anchored proteins such as CD55 and CD59 that partition into lipid raft domains as well as tetraspanins, proteins that are also found in these domains (Chaput et al. 2006). Exosomes also have distinct protein contents depending on their cell of origin (Fevrier and Raposo 2004; Stoorvogel et al. 2002).

The physiological consequences of exosomal secretion also differ according to the cell of origin. For instance Hsp70-containing exosomes are found in dendritic cells (DC), are enriched in major histocompatability class II complex molecules and appear to play a key role in cross priming and activating T lymphocytes (Chaput et al. 2006). Bone marrow-derived DC pulsed with acid-eluted peptides were shown to secrete immunogenic exosomes, mediate CTL responses in mice and lead to retardation of tumor growth (Zitvogel et al. 1998). Interestingly, the exosomes did not interact directly with T cells but required the mediation of the DC for cross priming and T cell activation. However, Hsp70-containing exosomes derived from tumor cells may have the opposite consequence in terms of tumor immunity depending on the cell type that they interact with. Tumor-derived exosomes from EL4 thymoma, TS/A mammary carcinoma and CT26 colon carcinoma cells were shown to be immunosuppressive on encountering and binding to myeloid-derived suppressor cells (MDSC) (Chalmin et al. 2010). The Hsp70 moiety found on the external surface of such tumor-derived exosomes was bound to Toll-Like Receptor 2 (TLR-2) and gave rise to signaling that led to activation of STAT3 in MDSC and down-regulation of tumor immunity *in vivo* (Chalmin et al. 2010). These investigators found that the Na^+/H^+ export channel inhibitor, amiloride, could inhibit exosomal release from tumor cells *in vivo* and reverse immune suppression (Chalmin et al. 2010). By contrast, when Hsp70 surface-positive exosomes, from tumor cells, interact with natural killer (NK) cells their migratory and cytolytic activities were shown to be activated, indicating immune stimulation (Gastpar et al. 2005). These different studies indicate the various properties of Hsp70-containing exosomes that depend on the nature of the cell of origin and the target cell. The exosomal response to stress has been termed the *stress-observation system* (SOS) and it has been proposed that export of Hsp70 in exosomes from stressed cells may be a form of intercellular communication that informs macrophages of cell stress and may arm such cells for innate immune responses (De Maio 2010). This group showed that treatment of HepG2 cells with heat shock at 43 °C leads to Hsp70 expression on the surface of the cells within lipid raft domains, leading to release of exosomes containing surface-orientated hsp70. Such vesicles were highly enriched in Hsp70 and when incubated with macrophages induced abundant secretion of tumor necrosis factor-α (Vega et al. 2008). The pro-inflammatory effectiveness of stress-induced exosomes depended on ongoing transcription and treatment of the Hep-G2 cells with actinomycin D led to inhibition of Hsp70 synthesis that correlated with loss of tumor necrosis factor-α inducing activity by exosomes. Overall therefore, secretion of Hsp70-containing exosomes is a widely-observed phenomenon with powerful immune and inflammatory consequences and can have a myriad of effects depending of the nature of the exosome-donor and acceptor cells.

7.5 Other Potential Mechanisms of HSP Secretion?

Another form of secretion utilized by cytokines is the IL-15 pathway (Duitman et al. 2008, 2010). IL-15 has the unusual property of being secreted while bound to its receptor, IL-15Rα. Such IL-15 ligand-receptor complexes are required for the IL-15 to passage through the ER and Golgi networks and be transported to the cell surface (Duitman et al. 2008, 2010). The presence of such complexes on the surface of fibroblasts, DC and monocytes then leads to expansion of NK and $CD8^+$ memory cell populations (Bergamaschi et al. 2008). One possible pathway for HSP release could therefore involve its coupling to known HSP receptors such as SRECI, LOX-1 and FEEL-1/stabilin-1 and transport of the molecular chaperones to the cell surface through the canonical secretion pathway (Calderwood et al. 2007). Again the question would arise—how could cytoplasmic HSP enter the ER or Golgi and bind to HSP receptors?

7.6 Triggers for HSP Release?

The proximal signal(s) leading to HSP sorting to the secretory compartments and release from cells are not known. Earlier studies showed that secretion of the DNA-binding protein, *high mobility group B1* (HMGB1), and the homeodomain protein *engrailed-2*, through the non-canonical pathway involves posttranslational modification (PTM). The triggering PTMs for these proteins are respectively, hyperacetylation and dephosphorylation (Bonaldi et al. 2003; Maizel et al. 2002; Mambula and Calderwood 2006a; Wisniewski et al. 1999). Hyperacetylation of HMGB1 may diminish its DNA binding affinity and permit nuclear exit prior to secretion. In the case of engrailed-2, phosphorylation within its homeodomain by casein kinase II deters secretion and this effect is reversed by dephosphorylation (Maizel et al. 2002). Interestingly, secretion of HMGB1 is attenuated during the heat shock response or when Hsp70 is overexpressed due to a block in nuclear export (Shi et al. 2006). There may, thus, exist cross-talk between proteins such as HMGB1 and Hsp70 secreted through the non-canonical pathways. It may also be significant that hyperacetylation of Hsp90α, although associated with lack of chaperone function, increases its binding to extracellular MMP-2 and enhances tumor invasion properties (Yang et al. 2008). The precise effects of these PTMs are not clear although they may involve loss of normal intracellular protein function, such as binding to DNA or chaperoning intracellular proteins, and may thus liberate the proteins to enter secretion pathways.

7.7 Conclusions

Non-canonical secretion of cytokines thus seems an enlightening paradigm for study of allied processes in molecular chaperone release. Like IL-1αβ secretion, release of Hsp70 appears to involve multiple independent pathways each supported by

impressive data, suggesting their independent involvement in delivering Hsp70 to the outside. Hsp70 for instance may enter the extracellular spaces either as free protein or packaged in exosomes and exert regulatory influences in its milieu. It is however not clear by which mechanisms Hsp70 crosses lipid membranes. Our studies on Hsp70 release during heat shock indicate that the chaperone is only secreted during the time of heating, suggesting that a triggering biophysical response to warming such as partial protein unfolding may occur (Mambula and Calderwood 2006b). This would be consistent with the idea of secretion of FGF and IL-1β during heat shock through a partially unfolded "molten globule" state that can cross membranes (Prudovsky et al. 2003). It may be significant that when cytoplasmic Hsp70 enters the lysosome in the process of chaperone-mediated autophagy or when it is transported into mitochondria, the protein is first unfolded in order to cross channels in the lipid membrane prior to refolding inside the organelle (Dice 2007; Pfanner and Truscott 2002). A similar unfolding process might trigger Hsp70 entry into secretory lysosomes or exosomal membranes in stress (De Maio 2010; Mambula and Calderwood 2006b; Mambula et al. 2007). The mechanisms through which HSP release is triggered under more physiological conditions are not so clear, although initiating steps could involve PTM of the HSPs, as with secretion of HMGB1 and engrailed-2.

We have concentrated in this review largely on HSP secretion through more physiological secretion mechanisms. However it is apparent that HSPs are also released when cells undergo necrosis (Mambula and Calderwood 2006a). Under these conditions Hsp70 for instance causes marked inflammatory effects that can be manipulated to mediate tumor rejection (Daniels et al. 2004; Kottke et al. 2007). Thus release of HSPs both from viable cells and from cells undergoing necrosis can have profound effects of immune and inflammatory responses.

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