Chapter 2
Discovery of the Extracellular Agonist Actions of Molecular Chaperones and Protein-Folding Catalysts

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Abstract Surprisingly, the history of the agonist actions of extracellular molecular chaperones can be traced back to the 1970s, with the cytokine macrophage migration inhibitory factor (MIF) and chaperonin (Hsp)10. The next cell stress protein to be identified as a molecular chaperone was the peptidylprolyl isomerase, cyclophilin A, in 1992. It is only later in the 1990s that the major signalling cell stress proteins—chaperonin (Hsp)60 and Hsp70 are found to have agonist activities. There are still ongoing discoveries of stress proteins with agonist actions and the latest such proteins are a new group of molecular chaperones—the extracellular/circulating molecular chaperones which include clusterin and α-acid1-glycoprotein.

2.1 Introduction

The phenomenon of the human system called science is endlessly fascinating for the paradoxes it encompasses. Science, at its heart, is the creation of the story of the Universe/Multiverse we live in, with its various disciplines focused on different parts of this larger picture. The basic unit of science is the testable hypothesis and the discoveries that the hypothesis machine provides us with are never complete and always need to be altered or even radically changed. Starting with the initial discovery of the heat shock response (Ritossa et al. 1962), the evolution of the phenomenon of the cell stress response and the discovery of the process of protein chaperoning (Laskey et al. 1978), it was the basic assumption that the proteins involved in the stress response were only active within the cell. This paradigm of molecular chaperones and protein-folding catalysts (PFCs—collectively cell stress proteins) being found only within the cell, has seriously, and negatively, influenced the new paradigm that has emerged since the 1990s, that molecular chaperones and PFCs can be secreted from the cell interior and function as cell surface receptors or cell signalling soluble agonists (See Chap. 1 for a personal history of the discovery of the secretion of Hsp70). This failure, by the cell stress protein community, largely to ignore the biology of extracellular molecular chaperones and PFCs, is curious, as in 1977, 1 year before Laskey's coining of the term chaperone for the function

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of nucleoplasmin, a group of Australian scientists identified an immunosuppressive factor in the serum of women in the first trimester of pregnancy (Morton et al. 1977; Noonan et al. 1979). Unfortunately, the molecular nature of this factor was not identified until 1994, when it was shown to be human chaperonin (Cpn)10 (Cavanagh and Morton 1994). However, this result seems to have been overlooked and the growing numbers of publications, arising from around the early 1990s, on the cell signalling actions of recombinant cell stress proteins, which would have been strongly supported by the physiological actions of Cpn10, were largely ignored or criticised on the grounds that they were due to bacterial contaminants (e.g. Tsan and Gao 2009). This criticism is still extant at the time of writing, even though there are a growing number of reports of the actions of extracellular cell stress proteins that could not possibly be due to bacterial contamination—either because the actions have nothing to do with the activity of pro-inflammatory bacterial contaminants or because the proteins under study are made in eukaryotic systems or are, indeed, totally synthetic proteins/peptides (Henderson and Pockley 2010; Henderson et al. 2010). It is hoped that reviews like this one, which provide a historical perspective, will encourage a fairer response to the study of the extracellular actions of molecular chaperones.

2.2 Secretion of Molecular Chaperones

Cell stress proteins are soluble signalling mediators—is a proposition that can only be accepted if there is evidence that these proteins are capable of being secreted. Again, the cell stress community has been guilty of ignoring key information, such as the early work from Tytel and Hightower that specific cell stress proteins are released from viable cells (Tytell et al. 1986; Hightower and Guidon 1989—see Chap. 1 for full details). The major problem has been a lack of understanding that both in bacteria (Holland 2010) and in eukaryotic cells (Nickel and Rabouille 2009), there are a plethora of protein secretion pathways in addition to the classical signal peptide secretion mechanism. Good evidence now exists that eukaryotic molecular chaperones can be secreted via one or other of these newly discovered secretion pathways (Table 2.1). In contrast, we know almost nothing about the release pathways that are involved in the secretion of bacterial molecular chaperones. Clearly much more work is needed to determine if the secretion of the many cell stress proteins found in the body fluids is due to a novel system for maintaining homeostasis, or if it is involved in tissue and cell pathology. However, it is now perfectly clear that a number of the major cell stress proteins are normally secreted and therefore their presence in body fluids and their actions on cells is not some artefact of the scientific process but is a manifestation of normal biological processes in both bacteria and eukaryotic cells.

Both bacteria and eukaryotes have a common set of cell stress proteins. It would appear, from the current literature, that eukaryotic cells have evolved to secrete numerically more of these proteins than bacteria have (Fig. 2.1). However, this may only represent the particular personal focus on the release of these proteins by both of these cell Kingdoms, with less attention being paid to bacterial cell stress proteins.

Table 2.1 The known secretion pathways for cell stress proteins

Protein	Secretion pathway	Reference
Thioredoxin	Novel pathway with some similarities to that of IL-1β	Rubartelli et al. 1990, 1992; Tassi et al. 2009
HSPB5	Exosomal secretion pathway	Gangalum et al. 2010
Peroxiredoxin	Brefeldin-insensitive non-classical pathway	Chang et al. 2006
PPIs	Unique vesicle-associated process	Suzuki et al. 2006
Cpn/Hsp60	Exosomal secretion pathway	Gupta and Knowlton 2007
Hsp70	Exosomal or vesicle-dependent secretion pathway	Lancaset and Febbraio 2005; Zhan et al. 2009
Hsp70	Non-classical pathway involving lysosomes	Mambula and Calderwood 2006
HspB1	Classic secretion pathway	Evdonin et al. 2009
BiP	Brefeldin-inhibited secretion pathway	Xiao et al. 1999
Hsp90	Possible exosomal pathway	Cheng et al. 2008

PPIs peptidyprolyl isomerases

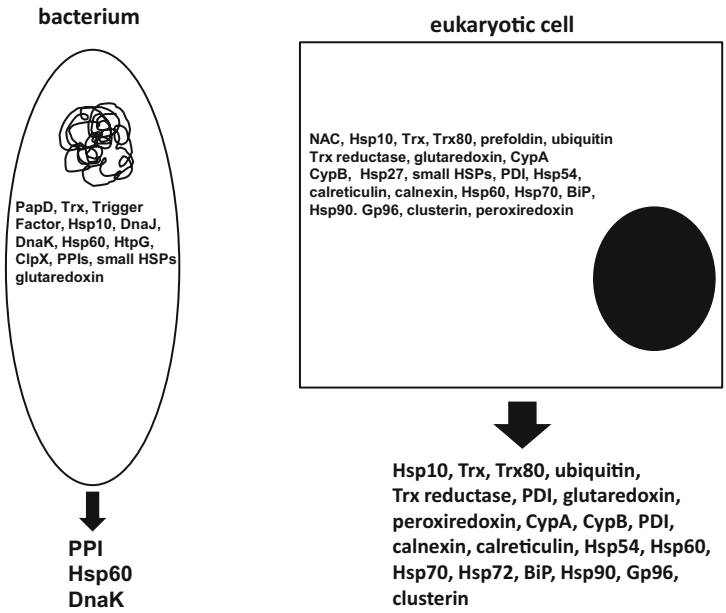

Fig. 2.1 The secretion of the total population of molecular chaperones and PFCs by bacteria and eukaryotic cells

2.3 Identification of the Cell Signalling Agonist Actions of Cell Stress Proteins

2.3.1 Chaperonin 10 (HSPE1)

As has already been discussed, the first evidence that cell stress proteins have the ability to act as cell signalling agonists was the report that a serum factor found in early pregnancy, and in consequence termed, early pregnancy factor (EPF), was an immunosuppressive factor (Morton et al. 1977). The nature of this factor was not clarified in the initial studies (e.g. Clarke et al. 1978) and it was only in 1979 that the immunosuppressive actions of this protein were defined (Noonan et al. 1979). However, it took until 1994 before the identity of EPF was confirmed. Using platelets as the source of EPF, led to the isolation of a pure form of this protein and its unequivocal demonstration as chaperonin (Cpn) 10, the co-chaperone for chaperonin (Cpn)60 (Cavanagh and Morton 1994). About a decade later it was shown that administration of recombinant human Cpn10 to rats could inhibit experimental inflammation (e.g. Zhang et al. 2003). Perhaps not surprisingly, this has led on to the Australian biopharmaceutical company AC Bio testing recombinant human Cpn10 for the treatment of a variety of human diseases including rheumatoid arthritis (Vanags et al. 2006) and multiple sclerosis (Broadley et al. 2009). One of the curiosities of the agonist actions of cell stress proteins is that it is impossible to predict what the next moonlighting action of these proteins will be. Proteins with more than one unique function are termed moonlighting proteins (Jeffery et al. 1999). It has recently been reported that Cpn10 is an erythropoietin-inducible secreted protein with effects on endothelial cell differentiation (Dobocan et al. 2009). Another interesting facet of cell stress proteins is the role that bacterial protein homologues play in disease. Thus with human Cpn10, the protein is immunosuppressive and anti-inflammatory. A similar situation is seen with the homologue from *Mycobacterium tuberculosis* (e.g. Ragno et al. 1996). In contrast the Cpn10 protein from the *Chlamydiae* appear to be pro-inflammatory (Zhou et al. 2011; Jha et al. 2011).

2.3.2 Macrophage Migration Inhibitory Factor (MIF)

While Cpn10 was the first molecular chaperone and cell stress protein to be discovered as a signalling agonist, the exact identification of the nature of EPF was not made until 1994. Thus, it can be argued that Cpn10 is not the signalling cell stress protein prototype. If this argument is heeded, then which cell stress protein takes the prize of being the first to be found with cell signalling activity? The surprising answer is that we can now take the history of cell stress proteins as immunomodulators back to 1971, and the discovery of the cytokine—macrophage migration inhibitory factor (MIF—e.g. Bartfield and Atoynatan 1971). This is one of the earliest and most confusing of cytokine molecules, which has been implicated in the pathology

of diseases including: sepsis, pneumonia, diabetes, rheumatoid arthritis, inflammatory bowel disease, psoriasis and cancer and for which novel low molecular mass inhibitors have been developed (Al-abed and Van Patten 2011). Whilst being around since the early 1970s, it was only in the late 1990s that the other guise of this protein was identified with the discovery that MIF is a thiol protein oxidoreductase (Kleeman et al. 1998; Potolicchio et al. 2003). A later study confirmed the influence MIF has on protein folding (Cherepkova et al. 2006). MIF is a secreted protein and it appears to induce its myriad effects by binding to CD74 on target cells, and such binding is now known to play a role in cancer (Shachar and Haran 2011). Unfortunately, nothing is really known about the connection between the protein-folding action of MIF and its myriad extracellular agonist functions. However, MIF serves to remind the reader that a potent cytokine can also function as a protein folding protein and should reinforce the hypothesis that molecular chaperones and PFCs can also function as cell signalling agonists with cytokine activity.

2.3.3 Thioredoxin

Again, the reader could accuse the writer that both examples of cell stress proteins so far discussed were only found to be molecular chaperones many years after their initial discovery as cytokine-like factors and that the earliest discovery of a molecular chaperone as a cell signalling agonist has not yet been defined. The example of the first molecular chaperone to be defined as a cell signalling agonist must then be human thioredoxin (Trx). This story can be said to start with the finding that lymphocyte activation is dependent on cell surface thiol oxidation status (Noelle and Lawrence 1981). Some years later it was reported that HTLV-1-transformed T lymphocytes secreted a factor, termed adult T cell leukaemia (**A**TL)-**d**erived **f**actor (ADF) that enhanced the expression of the p55 subunit of the IL-2 receptor. ADF synthesis was increased by classic T cell activators such as mitogens and phorbol ester. ADF was then shown to be human Trx (Tagaya et al. 1989). ADF was shown to be an autocrine growth factor for lymphocytes and to synergise with both IL-1 and IL-2 (Wakasugi et al. 1990). As has been shown in Table 2.1, Trx was the first protein folding catalyst to be shown to be secreted by a novel secretion pathway whose complete elucidation still eludes us. Interestingly, the secretion of key inflammatory cytokines such as IL-1 (Tassi et al. 2009) and the chemokine MCP-1 (Chen et al. 2010) is controlled by Trx. In addition to functioning to enhance the growth of T and B lymphocytes, Trx has also been shown to be secreted in greater amounts from T regulatory cells (T regs) and this is associated with a decreased level of T reg apoptosis (Mougiakakos et al. 2011).

Since the initial discovery of ADF/thioredoxin it has been established that Trx is a naturally secreted protein whose levels in the circulation are regulatable and with a wide range of important biological function (Holmgren and Lu 2010). This chapter is not the place to review the actions of thioredoxin and while elevated levels can be associated with poor outcome in patients with AIDS (Nakamura et al. 2001) it is

generally found that administration of this protein promotes beneficial effects in a variety of experimental animal disease states (Nakamura 2008). It is expected that this protein, or homologues of the protein, will find a place as therapeutics for a range of human diseases.

2.3.4 Peptidylprolyl Isomerases

These enzymes, which can be classified into the cyclophilins, FK506-binding proteins (FKBPs) and parvulins, are responsible for catalysing peptide bond *cis/trans* isomerisation (Schiene-Fischer et al. 2011). The cyclophilins (Cyps) and FKBs are intracellular proteins that bind to immunosuppressants, such as cyclosporine (Göthel and Marahiel 1999) and transduce their immunosuppressive actions. The first evidence that the cyclophilins had any signalling actions was the report that exposure of the mouse macrophage cell line, RAW267.4 to lipopolysaccharide (LPS), resulted in the release of cyclophilin (Cyp)A. The purified CypA exhibited pro-inflammatory activity *in vivo* and acted as a chemoattractant for human neutrophils and monocytes. This activity was blocked by cyclosporine A, but not by the structural analogue, cyclosporine H, which does not bind cyclophilin. The finding that CypA is released in larger amounts by LPS-stimulated than by non-stimulated macrophages and that the activity it exhibits is pro-inflammatory is identical to the situation with the secretion of true pro-inflammatory cytokines such as IL-1β and TNFα (Sherry et al. 1992). Thus this protein seems like it should be added to the group of pro-inflammatory cytokines.

Up to this date, all secreted cell stress proteins with agonist actions had been eukaryotic proteins. In the same year as mouse CypA was shown to be a pro-inflammatory factor it was reported that the essential *Legionella pneumophila* virulence protein, Mip (macrophage infectivity promoter), involved in the invasion of macrophages by this bacterium (Engleberg et al. 1989), was also a peptidylprolyl isomerase (Fischer et al. 1992). However, Mip was not a cyclophilin, but an FKBP (Engleberg et al. 1989). Other bacteria have also been found to utilise homologues of Mip to invade cells including *Chlamydia trachomatis* (Lundemose et al. 1993) and, coming more up to date, *Burkholderia pseudomallei* (Norville et al. 2011). In addition to being bacterial invasins, one of the peptidylprolyl isomerases of *Helicobacter pylori* is both a major immunogen in patients with gastric ulcers (Attanasov et al. 2002) and a signalling protein promoting major changes in the behaviour of both epithelial cells (Basak et al. 2005) and macrophages (Pathak et al. 2006).

Coming back to the human cyclophilin, if this protein had any role in disease then it would be found in relevant body fluids. This hypothesis was confirmed by the finding of elevated levels of CypA in the synovial fluid of patients with the inflammatory disease, rheumatoid arthritis, compared to those with the non-inflammatory condition, osteoarthrosis (Billich et al. 1997). Similarly, serum levels of peptidyl prolyl isomerase activity were elevated in patients with sepsis (Tegeder et al. 1997). Again, coming up to date, it has recently been shown that knock-out of the gene

encoding CypA renders mice unresponsive to acetaminophen-induced liver injury (Dear et al. 2011).

One of the major problems in the study of the biology of secreted cell stress proteins is identifying the nature of the agonist receptor and many such agonist proteins have no clearly identified receptor. This failure to identify single receptors for proteins such as Hsp60, Hsp70 and BiP has exacerbated the criticism that the effects of these proteins are artefactual. It is, however, satisfying, to record that secreted CypA and CypB have a well identified receptor in the protein, CD147 (Yurchenko et al. 2001). It is now established that the binding of CypA to CD147 is independent of the peptidyl prolyl isomerase active site (Song et al. 2011). It is reported that CypA and the chemokine CXCL2 have a cooperative influence in terms of neutrophil chemotaxis thus showing that the cell stress proteins can interact with cytokine networks (Heine et al. 2011). There is now significant evidence for the hypothesis that cyclophilin-CD147 interactions are pathogenic and this agonist-receptor pairing is now seen as an important therapeutic target for a range of human disease (Yurchenko et al. 2010).

2.3.5 Chaperonin (Hsp)60 (HSPD1)

Chaperonin (Cpn)60, generally known as heat shock protein (Hsp)60 in the human context, is one of the best studied secreted molecular chaperone agonists and one of the most heavily criticised, particularly the signalling activity of the human homologue. However, the discovery that this protein had signalling actions was made using the Cpn60.2 protein from the major human pathogen, *Mycobacterium tuberculosis* (Friedland et al. 1993). This paper revealed that exposure of the human macrophage cell line, THP-1 to recombinant *M. tuberculosis* Cpn60.2 induced the formation of a number of pro-inflammatory cytokines, including TNFα. The inference of this paper was that *M. tuberculosis* Cpn60.2, a protein better known at the time as Hsp65, was an activator of macrophages. This interpretation has had deleterious consequences for the field of secreted/signalling molecular chaperone biology. It was assumed that as this Cpn60 protein induced macrophage activation, then all homologues would do the same. The importance of this interpretation lies in the fact that the potent Gram-negative cell wall component, LPS, also stimulates ‘macrophage activation’. This left the Cpn60 protein open to the objection that its effect on macrophages was not due to an inherent biological activity, but to contamination with the LPS coming from the *E. coli* strains in which the Cpn60 proteins were expressed (Tsan and Gao 2009).

Macrophage activation is an oft-used term. In recent years, attempts have been made to subdivide this ‘activation’ into a number of distinct states (Gordon and Martinez 2010). Currently, macrophage activation, depending on which review is read, can be divided into: (i) classical (induced by gamma-interferon); (ii) innate (induced by bacterial components, principally LPS and; (iii) various forms of so-called alternative activation states (Gordon and Martinez 2010). Both classical and

innate activation involve the upregulation of genes encoding for proteins involved in bacterial recognition and bacterial killing. These would include, Fc gamma family proteins, MHC class II proteins and free radical inducing proteins. In addition, the classical and innate activators are major inducers of pro-inflammatory cytokines. It was therefore fascinating to find that when Ralph van Furth's group measured the effects of *M. tuberculosis* Cpn60.2 on monocytes that the major signs of macrophage activation, apart from cytokine synthesis, were missing (Peetermans et al. 1994). Thus, unlike LPS, the *M. tuberculosis* Cpn60.2 protein did not induce, for example, the synthesis of MHC class II proteins, required if the macrophage was going to present antigen to T lymphocytes. This Cpn60.2 protein is therefore inducing an activation state in macrophages which has not really been defined. However, what it is not doing, is mimicking the actions of LPS. Van Furth also showed that the *M. tuberculosis* Cpn60.2 protein activated human vascular endothelial cells in a manner distinct from that of LPS or human pro-inflammatory cytokines (Verdegaal et al. 1996). These early findings have been completely ignored by those workers who seek to criticise the study of the signalling actions of chaperonin 60 proteins.

Since these early studies of the signalling actions of the *M. tuberculosis* Cpn60.2 protein it has been shown that the Cpn60 proteins from *Aggregatibacter actinomycetemcomitans, E. coli, Mycobacterium leprae, Rhizobium leguminosarum, Chlamydia pneumoniae* and *C. trachomatis* and *Helicobacter pylori* can all stimulate macrophages and other cells to secrete cytokines (Henderson and Martin 2011). However, it was not until 1999, that it was shown that human Hsp60 would also induce both human and mouse macrophages to release pro-inflammatory cytokines (Chen et al. 1999). There is now a substantial literature on this protein which has been recently reviewed by Henderson and Pockley (2010).

In addition to stimulating monocytes, *M. tuberculosis* actually invades these cells and survives within them by subverting their anti-bacterial responsiveness (Rohde et al. 2007). It has been found that *M. tuberculosis* actually secretes large amounts of Cpn60.2 which adheres to the bacterial surface and acts as an adhesin for binding to macrophages. Binding is to the cell surface receptor CD43 (Hickey et al. 2009, 2010), a protein that is known to control the rate of intracellular growth of *M. tuberculosis* (Randhawa et al. 2008). Thus this Cpn60.2 protein plays multiple roles in allowing the bacterium to interact with macrophages. Further information on the secretion and role of circulating human Cpn60 will be provided by Pockley in Chap. 3.

2.3.6 Hsp70

The abbreviation Hsp70, now refers to at least twelve separate proteins, which are designated by a new nomenclature which was briefly described at the beginning of the book (Hageman et al. 2011). It was known since 1989 that Hsp70 could be secreted by cells (Hightower and Guidon 1989—reviewed in detail in Chap. 1) yet it was only in the twenty-first century that the first demonstration that Hsp70 could act as a stimulating agonist with human monocytes was published (Asea et al. 2000).

As with Hsp60, the nature of the receptor transducing the signal from Hsp70 has proved difficult to identify. Early studies, using cells transfected with Toll-like receptors (TLRs), suggested that TLR2/TLR4 were the receptors for Hsp70 (Asea et al. 2002). However, later studies from this group failed to replicate these findings and suggested that Hsp70 bound principally to the scavenger receptor LOX-1—a non-signalling cell surface protein (Thériault et al. 2005). A later study increased the number of scavenger receptors able to bind human Hsp70 (Thériault et al. 2006). Another proposed receptor for human Hsp70 is CD40 (Becker et al. 2002). Much more information on receptors for Hsp70 is to be found in Chap. 13 from Stuart Calderwood's group.

Some years after the discovery that human Hsp70 stimulated monocyte activation it was found that the Hsp70 (DnaK) protein from *M. tuberculosis* was also able to stimulate human monocytes to produce a range of key chemokines important in the pathogenesis of tuberculosis (Wang et al. 2001). The mycobacterial protein bound to CD40. This appeared to be the same as reported for the human homologue (Becker et al. 2002). However, the binding site for CD40 on the human and *M. tuberculosis* Hsp70 proteins is different. Thus the human protein binds via the amino-terminal nucleotide-binding domain in its ADP state (Becker et al. 2002). In contrast, the *M. tuberculosis* Hsp70 protein uses its C-terminal peptide binding domain to bind to CD40 (Wang et al. 2002). This suggests that the use of the CD40 receptor by the bacterial and eukaryotic Hsp70 proteins is an example of convergent evolution. In addition to binding to CD40, the *M. tuberculosis* Hsp70 protein also binds to the chemokine receptor and HIV-co-receptor, CCR5 (Floto et al. 2006).

2.3.7 Hsp27

Thus far all the cell stress proteins described have had the ability to stimulate leukocyte cytokine synthesis. In 2000, Carol Miller-Graziano and co-workers described the ability of human Hsp27 to promote human monocyte production of the anti-inflammatory cytokine, IL-10, relative to the production of modest levels of the pro-inflammatory cytokine, TNFα (De et al. 2000). This protein also blocks, by a unique mechanism, the differentiation of monocytes into dendritic cells (Laudanski et al. 2007). What is particularly interesting, is the actions of macrophages within tumours. Such tumor-associated macrophages (TAMs) are known to have major effects on tumour progression by producing factors that promote angiogenesis, remodel tissue and inhibit anti-tumoural immune responses (Muhktar et al. 2011). Miller-Graziano has reported that breast tumours produce very high levels of Hsp27, much of which is secreted. When exposed to Hsp27 monocytes are induced to become macrophages with immunomodulatory actions, having low levels of classic macrophage activation markers (HLA-DR, CD86 etc). These cells induce severe unresponsiveness and anergy in T lymphocytes and have very low tumouricidal activity but marked pro-angiogenic activity. These are the classic markers of the TAM and important in tumour growth (Banerjee et al. 2011). Thus it would appear that Hsp27, by controlling monocyte activation, can be a pro-tumoural protein.

1971	**Identification of macrophage migration inhibitory factor (MIF)**
1977	**EPF discovered as immunosuppressive factor in pregnancy**
1989	**Thioredoxin found to be a secreted lymphocyte modulator**
1992	**Cyclophilin A identified as pro-inflammatory secreted macrophage product**
1992	***Legionella pneumophila* virulence factor, Mif, involved in invasion of macrophages, shown to be a peptidyl prolyl isomerase**
1993	**First bacterial chaperone, chaperonin 60, shown to be an inducer of cytokine synthesis**
1994	**EPF shown to be chaperonin 10**
1999	**MIF shown to be a chaperone**
1999	**Human Hsp60 shown to stimulate macrophage cytokine synthesis**
2000	**Human Hsp70 shown to stimulate macrophage cytokine synthesis**
2001	**Hsp27, first example of a monocyte-deactivating chaperone**
2001	**BiP, a human ER chaperone also found to have anti-inflammatory mode of action**

Fig. 2.2 Timeline of the discovery of the signalling activity of cell stress proteins

To provide the reader with a visual representation of the history of the cell stress protein as a signalling agonist a time line has been provided (Fig. 2.2).

2.4 Conclusions

To paraphrase an ancient saying. ‘always expect the unexpected’. This is the situation in the field of cell stress proteins, with a growing number of such proteins exhibiting a myriad of biological actions over-and-above the facility to promote correct protein folding. These proteins can now be defined as moonlighting proteins—that is—proteins with more than a single unique function (Jeffery 1999). Currently around

16 human cell stress proteins and 4 bacterial proteins have been found to moonlight, largely as immune modulators with agonist properties. It is predicted that all molecular chaperones and protein folding catalysts will have the ability to be expressed on the surface of cells and/or to be secreted and thus to signal. This (the author postulates) allows the key homeostatic mechanism, the cell stress system, to broadcast to other cells and tissues that there is some form of stressor in the vicinity allowing nearby cells to prepare for it. It will be of interest to see if this hypothesis can survive a full-blown Popperian test.

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