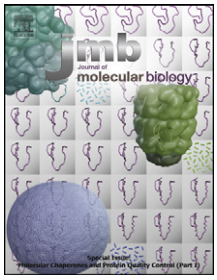
Review



BiP and Its Nucleotide Exchange Factors Grp170 and Sil1: Mechanisms of Action and Biological Functions

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http://dx.doi.org/10.1016/j.jmb.2015.02.011
Edited by J. Buchner

Abstract

BiP (immunoglobulin heavy-chain *bi*nding *p*rotein) is the endoplasmic reticulum (ER) orthologue of the Hsp70 family of molecular chaperones and is intricately involved in most functions of this organelle through its interactions with a variety of substrates and regulatory proteins. Like all Hsp70 family members, the ability of BiP to bind and release unfolded proteins is tightly regulated by a cycle of ATP binding, hydrolysis, and nucleotide exchange. As a characteristic of the Hsp70 family, multiple DnaJ-like co-factors can target substrates to BiP and stimulate its ATPase activity to stabilize the binding of BiP to substrates. However, only in the past decade have nucleotide exchange factors for BiP been identified, which has shed light not only on the mechanism of BiP-assisted folding in the ER but also on Hsp70 family members that reside throughout the cell. We will review the current understanding of the ATPase cycle of BiP in the unique environment of the ER and how it is regulated by the nucleotide exchange factors, Grp170 (*g*lucose-*r*egulated *p*rotein of *170* kDa) and Sil1, both of which perform unanticipated roles in various biological functions and disease states.

Protein Folding and Quality Control in the ER

Approximately one-third of the human genome encodes proteins that reside at the cell surface, that are secreted, or that populate organelles of the secretory pathway. These proteins are synthesized at the endoplasmic reticulum (ER) membrane and are translocated into the lumen where they acquire their functional tertiary and quaternary structure. The folding of these proteins and their assembly into larger heteromeric complexes is guided by the same principles and processes used throughout the cell, but it is further complicated by the addition of large branched oligosaccharide moieties to nascent chains entering the ER, high ER concentrations of calcium, and an oxidizing environment combined with systems that catalyze the formation of intrachain and interchain disulfide bonds. As a result, tight quality control measures have evolved to monitor the success of secretory pathway protein maturation, a process known as ER quality control (ERQC), which is conserved in most eukaryotic organisms (Fig. 1a and b). If a newly synthesized protein folds properly and passes the scrutiny of the ERQC machinery, it can undergo vesicular-mediated transport through the organelles of the secretory pathway (Fig. 1c), but if the protein fails this inspection, it must be targeted for proteasomal degradation in the cytosol *via* a process known as ER-associated degradation (ERAD) (Fig. 1d). A tremendous number of studies have led to the identification of many of the proteins involved in these two outcomes and have provided a detailed understanding of their underlying mechanisms. At the heart of this triage decision are two major chaperone systems: the lectins calnexin/calreticulin (Fig. 1a), which are unique to the ER, and the Hsp70 system (Fig. 1b), which has many aspects common to all Hsp70s. BiP (immunoglobulin heavy-chain *bi*nding *p*rotein) is the only conventional Hsp70 chaperone in the ER, but a related large Hsp70 protein with chaperone activity, Grp170

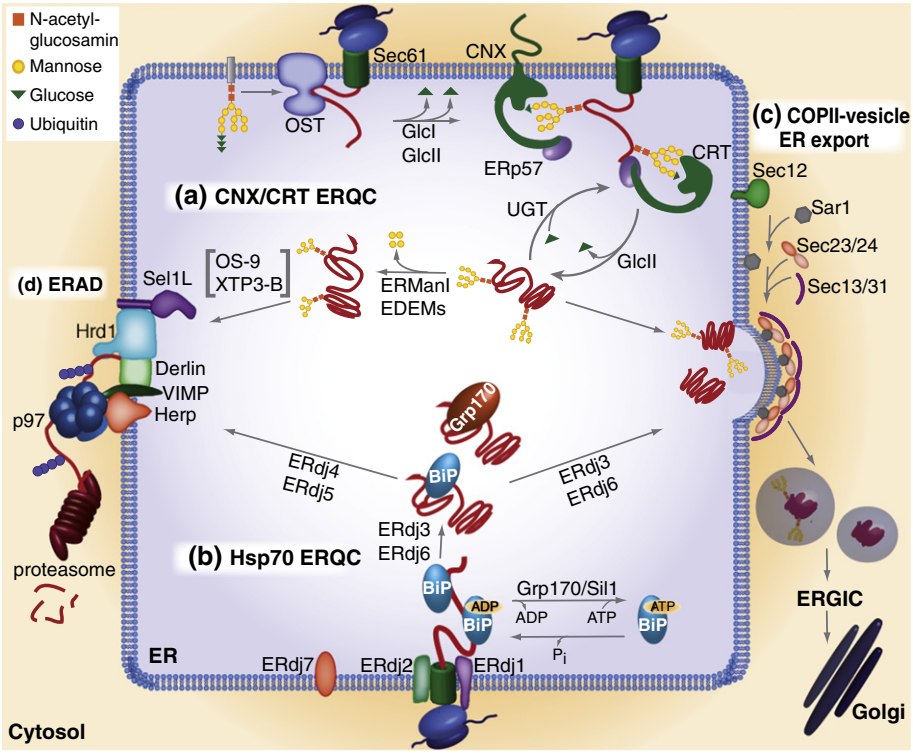

Fig. 1. The ERQC machinery. Two main chaperone systems, the lectins calnexin/calreticulin (CNX/CRT) (a) and the Hsp70 chaperone BiP (b), aid the folding proteins for secretion (c) or target them for ERAD if folding fails (d). (a) The oligosaccharyl transfer (OST) complex attaches a core oligosaccharide from a dolichol donor to the Asn of the Asn-X-Ser/Thr motif on nascent proteins during their translocation into the ER. GlcI (glycosidase I) and GlcII (glycosidase II) remove the outer two glucose residues of the oligosaccharide, allowing the remaining glucose to be recognized by CNX/CRT. CNX/CRT assists protein folding in concert with further co-chaperones such as the family member, ERp57. Proteins exit the CNX/CRT cycle once the last glucose residue is removed by GlcII. If folded properly, the protein is released from the lectin chaperone cycle and is transported further along the secretory pathway. Incompletely folded intermediates can re-enter the CNX/CRT cycle if a single glucose is re-attached by the folding sensor UDP-glucose glycoprotein glucosyltransferase (UGT). If folding ultimately fails, proteins are further trimmed by ERManI (*ER man*nosidase *I*) and/or an EDEM (*ER d*egradation *e*nhancing α-*m*annosidase-like protein) resulting in removal of 4 mannose residues and recognition by OS-9 (*o*steo*s*arcoma amplified *9*) and XTP3-B (*XTP3* transactivated protein *B*), which then transfer the trimmed glycoprotein to the ERAD machinery for disposal. (b) The Hsp70 chaperone BiP binds hydrophobic patches exposed on nascent or incompletely folded proteins that are often non-glycosylated. BiP possesses low substrate binding affinity in the ATP-bound state and high affinity upon hydrolysis of ATP to ADP. Grp170 and Sil1 facilitate substrate release from BiP by stimulating the release of ADP and allowing ATP to rebind and open the lid on the SBD. Seven ERdj co-factors that interact with BiP *via* their J-domain and assist BiP in its functions during protein translocation (ERdj2), protein folding (ERdj3 and ERdj6) and ERAD (ERdj4 and ERdj5) have been identified. The functions of ERdj1 and ERdj7 are not well understood, nor is the role of the large Hsp70, Grp170, that also binds to some incompletely folded BiP client proteins. (c) Once the threshold of folding set by the ERQC is met, proteins exit the ER in COPII (*co*at *p*rotein complex *II*)-coated vesicles, a process that is initiated by Sec12 and driven by a GTPase, Sar1, and four major coat proteins, Sec23, Sec24, Sec13, and Sec31. (d) Once proteins that are clients of either chaperone system are delivered to the ERAD machinery, their retrotranslocation into the cytosol is facilitated by a complex of several transmembrane proteins including Sel1, Der1-like proteins, VIMP (*v*alosin-containing protein/p97-*i*nteracting *m*embrane *p*rotein), Herp, and Hrd1 (*h*ydroxymethyl-glutaryl *r*eductase *d*egradation protein *1*), which connect the machinery in the ER lumen to the protein ubiquitination machinery in the cytosol, allowing the ERAD client to be recognized by the p97 hexameric ATPase in the cytosol that provides the energy for extracting a protein from the ER for degradation by the 26S proteasome.

(*g*lucose-*r*egulated *p*rotein of *170* kDa), is also present. Unlike the lectins, which monitor both N-linked glycans and unfolded regions on nascent polypeptide chains [1], BiP detects only the latter and is the major system used for non-glycosylated proteins or for some glycoproteins in which the most N-terminal glycan occurs relatively late in the linear sequence [2]. The ability of BiP to bind and release unfolded protein substrates is tightly regulated by a cycle of ATP binding, hydrolysis, and nucleotide exchange, which is controlled by a number of co-factors. They include seven ERdjs

(*ER*-localized *D*na*J*-like protein*s*), several of which bind directly to unfolded proteins and transfer them to BiP before stimulating its ATPase activity [3,4], and two nucleotide exchange factors (NEFs) that trigger the release of bound substrates [5,6]. Recent data suggest the ERdj co-chaperones play distinct roles in regulating BiP's involvement in functions that lead to disparate fates for the bound client. These insights have extended our understanding of how BiP's ATPase cycle is regulated, which together with the NEFs Grp170 and Sil1 is the focus of this review.

The ATPase Cycle of BiP in the ER Environment

A resting state of BiP

Changes in the external environment or different developmental stages of a cell can result in large variations in the load of unfolded or misfolded proteins in the ER. BiP as one of the major ER chaperones must therefore be readily and rapidly available in times of need. Differentially modified and assembled forms of BiP seem to be present in the ER to cope with altering cellular conditions. In the absence of stress, BiP has been shown to be a major ADP-ribosylated protein in mammalian cells [7] and is thought to be phosphorylated [8,9]. However, whenever the load of unfolded proteins increases in the ER, the amount of modified BiP decreases [7,9–11]. Accordingly, it has been suggested that the ADP-ribosylated or phosphorylated forms of BiP represent a pool of inactive oligomeric molecules that can quickly be reactivated when needed [7,12,13] (Fig. 2a). In agreement with this idea, only monomeric, unmodified BiP is bound to substrates [9,13]. Despite this and other [14–16] circumstantial evidence suggesting that BiP's activity *in vivo* might be affected by post-translational modifications, it remains unclear how these modifications are controlled in response to physiological cues. Although neither the kinase nor ADP-ribosyltransferase has been identified, the ADP-ribosylation site of BiP was recently mapped to two Arg residues within its substrate binding domain (SBD) [11]. Modification of these residues would interfere with substrate binding and thus explain earlier observations that only unmodified BiP is bound to substrates. Substrates and ATP can induce dissociation of BiP oligomers [15,17], thereby reshuffling BiP into its functional ATPase cycle in the ER. As mutation of these two Arg residues abolished all post-translational modifications of BiP, the role of phosphorylation has been questioned [11]. Very recently, two groups reported that the nucleotide binding domain (NBD) of *Drosophila* [18] and human [19] BiP can be modified by AMPylation. Both studies found that the enzymes responsible for the modification, dFIC and HYPE, respectively, were induced by ER stress, although the effect of this modification on BiP activity is presently inconclusive. Importantly, possible effects of the various modifications on the interaction of BiP with ERdj co-factors and NEFs have not been determined.

The ATP-bound state of BiP

Like all Hsp70s, BiP is an ATP-dependent molecular chaperone [20–23]. A large variety of BiP mutants, recent single-molecule studies, and a plethora of data on other Hsp70 family members now allow us to describe the ATPase cycle of BiP in quite some detail (Fig. 2). Once ATP binds, BiP enters a state of low substrate affinity with high on and off rates for substrate binding [23,24]. A complex allosteric mechanism transmits information on the nucleotide state from the NBD to the SBD and on the substrate occupancy state from the SBD back to the NBD [25]. This reciprocal information transfer is best understood for the *Escherichia coli* Hsp70 DnaK [26–29]. ATP binding leads to a closure of the otherwise quite dynamic NBD around the nucleotide [30–35]. The ATP binding status is then transmitted to the SBD and leads to an opening of the substrate binding cavity by increasing SBD flexibility and lid opening [35,36]. It is still not entirely clear exactly how the nucleotide binding state is transmitted from the NBD to the SBD. Different signal transmission pathways seem to exist between these two domains in the Hsp70 molecule, and details of these pathways may vary for different Hsp70 family members [26–29,37,38]. However, three different themes have emerged for how interdomain communication occurs. In DnaK, bound nucleotide is sensed by residues in the NBD that lie in close proximity to the bound ATP, and this information is thought to be transmitted *via* a Pro residue, which can likely undergo *cis*/*trans* isomerization, to an interface at the surface of the NBD that interacts with the SBD [39]. Interestingly, all residues of the proposed DnaK sensor-relay system are conserved in BiP [30,31]. However, the impact of mutations of the corresponding residues has not been investigated in BiP. In addition to this putative Pro-focused sensor-relay system, Thr37 in the NBD plays a particularly important role as a nucleotide sensor in BiP [40], likely due to a direct interaction of its hydroxyl group with the γ-phosphate oxygen of the bound ATP [41]. How Thr37 signals to the surface of the NBD is not currently known, but it has been shown that once ATP binding is transmitted to the NBD surface, it influences the interaction of positively charged residues on the NBD with negatively charged residues on the opposing surface of the SBD [39,42]. The third known relay that signals nucleotide binding from the NBD to the SBD occurs through the conserved hydrophobic linker, which connects the

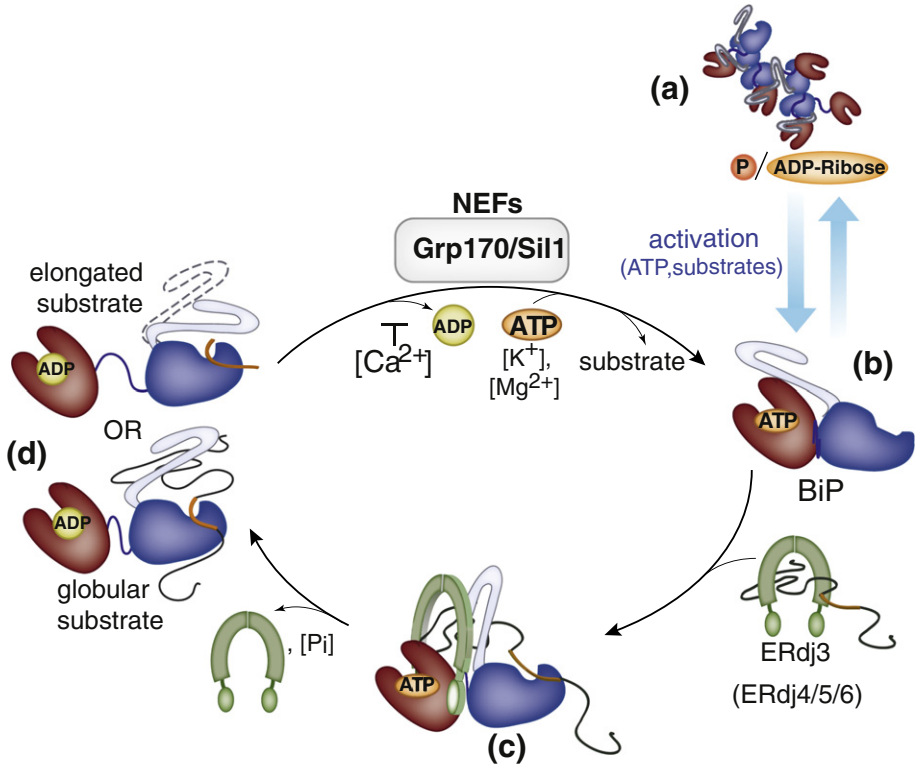

Fig. 2. The ATPase cycle of BiP in the ER. (a) In the absence of high substrate loads, BiP exists in a multimeric form that is post-translationally modified (ADP-ribosylated and perhaps phosphorylated), which renders the protein inactive. When the demand for BiP increases, the modifications are removed allowing a readily accessible pool of BiP to be reactivated. (b). Once BiP binds potassium and ATP, its NBD (red) and its SBD (blue) come into close proximity to each other and the lid of the SBD (grey) opens, which results in a form that binds substrates with low affinity. (c) Substrates can be introduced into the BiP cycle *via* their initial binding to DnaJ-like co-chaperones such as ERdj3 (green), which transfers substrate to BiP and increases BiP's ATPase activity thereby locking the substrate onto BiP. Note that the binding sites within the substrate for ERdj3 and BiP (shown in orange) are probably not identical allowing a transient three-way complex, which has been detected both *in vivo* [196] and *in vitro* [48]. (d) After the magnesium-dependent hydrolysis of ATP, BiP enters a state with low on and off rates for substrates. For elongated/peptide substrates, the lid closes over the bound substrate, whereas direct interactions between the lid and the substrate exist for globular substrates but the lid may not close completely. The SBD and NBD become more distant upon substrate binding and ATP hydrolysis, which is less pronounced for globular substrates. ADP must be exchanged against ATP in order to release the substrate and make BiP available for another round of client binding. Calcium increases the affinity for ADP, whereas the NEFs Grp170 and Sil1 facilitate the nucleotide exchange reaction.

two domains. Upon ATP binding, the linker binds to a cleft in the NBD, which is important in transmitting the nucleotide state of the NBD to the SBD and increases ATP hydrolysis of the NBD once bound to the cleft [35,36,43,44]. Although some details of the NBD–SBD interface and regulation by further co-factors vary for different Hsp70s [36,45,46], the combination of these conserved allosteric signaling pathways, which are most likely interconnected, results in a compaction of the NBD and SBD onto each other and an opening of the SBD lid upon ATP binding [27,37,47,48] (Fig. 2b). Of note, ATP hydrolysis itself is not necessary for the conformational changes leading to the opening of the SBD, but binding of ATP in combination with potassium ions is sufficient for these changes [40,49,50]. Very important contributions toward understanding the nucleotide-induced conformational changes in Hsp70 molecules came from two recent crystal structures of the ATP--bound form of DnaK [51,52]. Both studies show that, upon ATP binding, the lid of the SBD docks onto the lobe I side of the NBD. In addition, the SBD itself docks onto an interface made up of lobes I and II of the NBD, and the NBD–SBD linker binds to a small crevice at the base of the two lobes. These binding events are transmitted to the SBD where the substrate binding channel opens up and, together with increased flexibility [51] or even complete opening of the outer loops of the SBD [52], allows substrate release to occur upon ATP binding [53].

ATP hydrolysis and the ADP state

Like most Hsp70 proteins, BiP has a very low intrinsic, Mg^{2+}-dependent ATPase activity [23,54]. Once ATP is hydrolyzed, the SBD–NBD interface is broken and both domains behave more independently of each other. The degree of domain separation in the ADP state seems to vary for different Hsp70s. In DnaK, the SBD and NBD act independently when in the ADP state with only transient contacts between these two domains [36,47,55,56]. Conversely, in the case of a bovine Hsc70, extensive contacts between the SBD and the NBD are detected even in the ADP state [46], whereas single-molecule measurements on mitochondrial Hsp70 [47] and BiP [48] argue for a heterogeneous ensemble of conformations. Taken together, in the ADP state, the NBD and SBD seem to be in a dynamic distance distribution with a general trend toward domain separation. Finally, not only the distance between the SBD and NBD appears to vary in the ADP-bound state of Hsp70s but also the lid likely populates open and closed conformations with a general trend toward more closed conformations, as demonstrated for DnaK, mitochondrial Hsp70, and BiP [47,48,57]. Thus, in contrast to the ATP state characterized by close NBD–SBD contact and an open lid, the ADP state seems to be more heterogeneous within a single type of Hsp70, as well as more diverse between individual Hsp70s.

Substrates in the ATPase cycle of BiP

The binding of substrates has a very interesting impact on the ATPase cycle of Hsp70 proteins. It has long been appreciated that binding of peptide substrates accelerates the ATP hydrolysis rate of BiP [40,58] and other Hsp70s [26–29,37,38]. Most studies have been performed with small hydrophobic peptides that were thought to be good substrate mimics. In the case of BiP, doubt has been cast on the significance of these results, since a completely unfolded BiP-binding protein did not stimulate BiP's ATPase activity in contrast to short peptides [54]. Indeed, the only known sources of small peptides in the ER are those that enter through TAP (*t*ransporter associated with *a*ntigen *p*rocessing) transporters to be delivered to MHCI (major histocompatibility complex class I) molecules [59] and signal peptides that may never fully enter the ER lumen, neither of which has been shown to interact with BiP. Thus, it is more likely that the substrates BiP encounters are elongated polypeptide chains that are in the process of translocation *via* the Sec61 channel, proteins that are partially folded, as well as misfolded or even aggregated proteins. In addition, BiP binds to native forms of Sec61 to gate the translocon [60,61] and the UPR (unfolded protein response) transducers to maintain them in an inactive form [62,63]. Presumably, this involves unfolded or unstructured loops or regions in these proteins that allow them to interact with BiP in a regulated manner. Recent work has shown that, in the case of a more globular substrate, in contrast to what has been observed for peptides, complete lid closing over the bound substrate does not necessarily occur [48,57]. In this case, direct interactions between the lid and the substrate were observed, leading to even further extension of the conformational space available within the Hsp70s' SBD. This direct lid–substrate interaction, as well as the recently described interaction between the unstructured C-terminus of Hsp70 chaperones and their substrates [64], might aid in conformational remodeling of Hsp70 clients. The ATP-induced opening of the lid [51,52] and the inherent dynamics observed for the lid [48,57] might also have an impact on substrate conformations if lid–substrate interactions occur. Of note, when a peptide binds to the ATP state of DnaK, it shifts the structure of this chaperone more toward the ADP state [36], whereas binding of a larger polypeptide substrate to the ADP state of BiP induces a conformation that more closely resembles the ATP state in terms of NBD–SBD separation and lid opening [48] (Fig. 2d). Thus, the substrate itself seems to shift the conformational state of the Hsp70s, playing an active role in the allosteric signaling mechanism. As substrates apparently shift Hsp70s structurally away from the pure nucleotide-regulated states, they might facilitate transitions between them. Important insights into the structural transitions within the Hsp70 DnaK upon nucleotide and substrate binding have recently been obtained by a comprehensive NMR study [65]. An intermediate between the fully undocked NBD–SBD conformation (ADP-bound state) and the completely docked conformation (ATP-bound state) of DnaK was identified, which showed disruption of the interactions between the NBD and the SBD while the binding of the linker to the NBD was retained. Of note, substrate was shown to induce this intermediate, revealing how substrates can directly influence the conformational transitions within an Hsp70 molecule. This highlights the energetic competition between the different possible states of Hsp70s, which vary from Hsp70 member to Hsp70 member [36,46–48,55,56,65] and allow extrinsic and intrinsic functional fine-tuning of the ATPase cycle.

In the cell, substrate transfer to Hsp70s is regulated by DnaJco-chaperones that can further increase the ATPase rate of the Hsp70 molecule and thereby lock a substrate on the Hsp70 molecule in its ADP state [28,66,67]. Thus, in the ER, ERdj proteins can help define where substrates are bound by BiP, how fast substrates are bound, and even in the selection of which substrates are bound [68]. Indeed, some ERdjs directly bind to substrates and deliver them to the Hsp70 molecule [4,68] (Fig. 2c).

Mechanistically, they might shift the Hsp70 molecule toward a pre-hydrolysis conformation, perhaps by destabilizing the aforementioned charged SBD–NBD interface, thus facilitating ATP hydrolysis. Alternatively, they might either increase interaction of the hydrophobic linker with the NBD or act as a linker mimetic themselves thereby accelerating ATP hydrolysis [42,46,69]. Conflicting models exist for the binding of DnaJco-chaperones and the detailed molecular mechanism of their action is still unclear [36,45,46,69] and may vary between different Hsp70s and their DnaJ co-factors. For BiP, it has been shown that ERdj3, a major co-chaperone in *de novo* protein folding in the ER [4], opens up the lid of the SBD thereby facilitating binding of a substrate protein [48]. Conversely, ERdj3-induced lid opening results in release of small bound peptides, underscoring the difference between peptides and proteins as Hsp70 substrates [48]. This shows that an ERdj protein can directly alter the lid conformation of BiP to poise it for binding to more globular folding intermediates states, which are likely to be encountered in the lumen of the ER.

Re-entering the ATPase cycle

BiP has to be refueled with ATP in order to release a bound substrate and enter a new round in the ATPase cycle. The proper functioning of the BiP ATPase cycle is crucial for substrate maturation in the ER [70,71] and the integrity of the entire organelle [72,73]. It might thus come as no small surprise that the conditions of the ER are far from optimal for an efficient ATPase cycle of BiP. The optimal pH for BiP's ATPase activity is quite acidic, and at a more physiological ER pH of around 7, the intrinsic ATPase of BiP is particularly slow [23]. Even more striking, the presence of calcium, which is present at high concentrations in the ER, almost completely inhibits the ATPase activity of BiP *in vitro* [23,74]. Calcium has been shown to increase the affinity of BiP for ADP almost 1000-fold, thereby inhibiting nucleotide exchange and accordingly BiP's ATPase activity [75] (Fig. 2). In contrast to an *in vitro* setting, where hydrolysis is the rate-limiting step in the ATPase cycle of BiP [54], nucleotide exchange is thus most likely rate limiting in the ER. Calcium might even poise BiP to enter an inactive phosphorylated state [10]. Indeed, substrates are not readily released from BiP *in vivo*, suggesting that BiP does not seem to continuously traverse through its ATPase cycle [76]. Thus, the chemical environment of the ER, the need for regulated substrate release, and maybe even the reactivation of BiP from its resting state render another class of accessory proteins for BiP completely indispensable: the NEFs, Grp170 and Sil1. NEFs release ADP allowing ATP to rebind (Fig. 2), thereby decisively influencing how fast substrates are released from BiP, where release in the ER occurs, and maybe even in which folding state substrates are released from BiP.

Regulating the ATPase Cycle of BiP in the ER Environment by Sil1

The identification of a NEF for the ER lumenal Hsp70

Although BiP's ATPase cycle would appear to be particularly dependent on co-factors to regulate its binding and release from substrates, no resident ER proteins that possessed nucleotide exchange activity had been identified in any organism until the late 1990s (see Fig. 3 for an overview of cytosolic and ER lumenal Hsp70 NEFs; cytosolic Hsp70 NEFs are reviewed in Ref. [77]). Then several laboratories independently identified the first BiP NEF in three different organisms. Sls1p was identified in a synthetic lethal screen to identify genes that interacted with the SCR2-encoded 7S RNA component of SRP in the yeast *Yarrowia lipolytica* [78,79]. It was induced by both heat shock and ER stress and was shown to be a lumenal ER protein that interacted with both Kar2p (yeast BiP) and Sec61, a component of the translocon. Deletion of *SLS1* dramatically decreased the synthesis of secretory pathway proteins leading to the hypothesis that Sls1p played a role in the translocation of proteins into the ER lumen. Subsequently, an Sls1p homologue was identified in *Saccharomyces cerevisiae* and shown to bind preferentially to the ADP-bound form of yeast BiP. It stimulated BiP's ATPase activity in the presence of a DnaJ-like co-factor, Sec63p, by accelerating ADP release, thus making Sls1p the first NEF to be identified for BiP [80–82]. Independently, studies were being conducted to determine the function of Lhs1p (*l*umenal *H*sp *s*eventy), an Hsp70-related chaperone found in the yeast ER that is the homologue of mammalian Grp170 [83]. *S. cerevisiae* strains that were null for Lhs1p were viable but had a constitutively induced UPR. Blocking UPR activation by constructing a *Δlhs1Δire1* mutant resulted in a severe growth defect. A multicopy suppressor screen was conducted to identify genes whose overexpression would silence the severe growth arrest observed in the *IRE1*, *LHS1* double deleted strains [83]. This led to the identification of *SIL1*, which was identical with *SLS1*. The *Δlhs1Δsil1* mutant was lethal, leading the authors to suggest that both Sil1p and Lhs1p might play a similar role in regulating Kar2p's activity [83]. Meanwhile, the ATPase domain of human BiP was used as bait in a two-hybrid screen of a human liver cDNA library to identify proteins that might regulate its activity [5]. This approach probably would not have

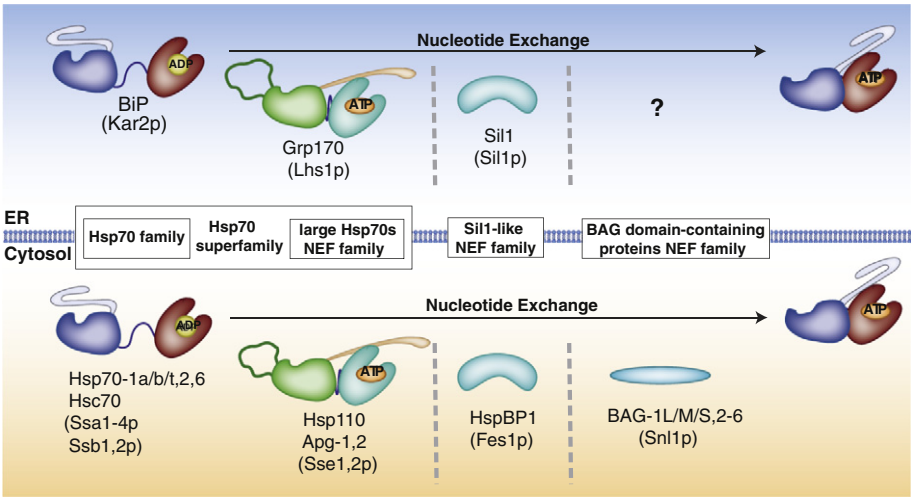

Fig. 3. ER resident and cytosolic Hsp70 family members and their NEFs. Several members of the Hsp70 protein family exist in the cytosol (bottom) of eukaryotic cells (yeast homologues are indicated in brackets), whereas a single member, BiP, resides in the ER (top). Three different classes of Hsp70 NEFs have been identified in the cytosol: large Hsp70 family members, Sil1-like, and Bagdomain proteins that remove ADP from the NBD of Hsp70s, thus allowing ATP to bind and substrates to be released. A single member of the first and second class of NEFs, Grp170 and Sil1, respectively, has been found to act as NEFs for BiP in the ER. While Sil1-like and Bag-domain-containing proteins are structurally unrelated to Hsp70 proteins, the large Hsp70 family of NEFs share many structural features with conventional Hsp70s. The Hsp70 superfamily comprises conventional and large Hsp70s.

succeeded except for a bit of serendipity. Although the bait vector was constructed with both a wildtype and a mutant BiP ATPase domain, the screen was conducted first with the mutant ATPase domain. The BiP-associated protein (BAP) was identified and demonstrated to bind preferentially to a variety of BiP ATPase mutants in the mammalian ER. When the wild-type BiP ATPase domain was used as bait, it failed to produce yeast colonies when co-expressed with BAP, suggesting that BAP preferred a particular conformation or nucleotide-bound state of BiP. Similar to studies with Sls1p [80], BAP was found to interact preferentially with the ADP-bound form of BiP and stimulate nucleotide release, thus driving the ATPase cycle forward [5]. From this point forward, we will refer to the proteins of all three species as Sil1/Sil1p.

Mechanism of exchange activity

Amino acid sequence comparisons revealed that yeast and mammalian Sil1 shared significant but low identity with cytosolic Fes1p and HspBP1 (*Hsp*70 *b*inding *p*rotein *1*), respectively. Data were beginning to accumulate at this time, demonstrating that these cytosolic proteins regulated the ATPase activity of their respective Hsp70s [82,84], even though they bore no apparent structural relationship to the only previously identified Hsp70 NEFs: *E. coli* GrpE [85] or eukaryotic cytosolic Bag domain proteins [86]. Both GrpE and Bag-1, although structurally distinct themselves, bind to the cleft at the top surface of the Ib and IIb subdomains of DnaK/Hsp70 NBD and in a similar fashion "push" the IIb domain away from the Ib domain, thereby allowing nucleotide to be released (reviewed in Refs. [26] and [77]). The structure of human HspBP1 was solved with only domain II of the Hsp70 NBD. It revealed that HspBP1 is composed of four armadillo-like repeats that wrap around subdomain IIb from the side and bind to it with a higher apparent affinity than Bag-1 [87]. When this structure was superimposed on the structure of the Hsc70 NBD crystallized with ADP [32], it became clear that this orientation of HspBP1 would have severe steric conflicts with domain Ib in the intact NBD. Thus, the data suggested that HspBP1 served not only to push the IIb subdomain away from Ib, like GrpE and Bag-1 do, but was likely to also displace the Ib lobe, thus further destabilizing this domain and decreasing its affinity for nucleotide [87]. This argued that, unlike Bag-1, HspBP1 induced a major distortion of the Hsp70 ATPase domain to trigger nucleotide release. More recently, a structure of yeast Sil1p was solved with the complete NBD of Kar2p [88]. Similar to HspBP1, the Sil1 protein is composed of four armadillo motifs that wrap around the IIb lobe of the BiP ATPase domain (Fig. 4a). Importantly, this structure further revealed that Sil1p also makes contact with subdomain Ib causing lobe IIb, and to a lesser extent Ib, to rotate away from the bound nucleotide concomitant with its release (Fig. 4b), which confirmed the hypothesis put forward for the HspBP1 structure.

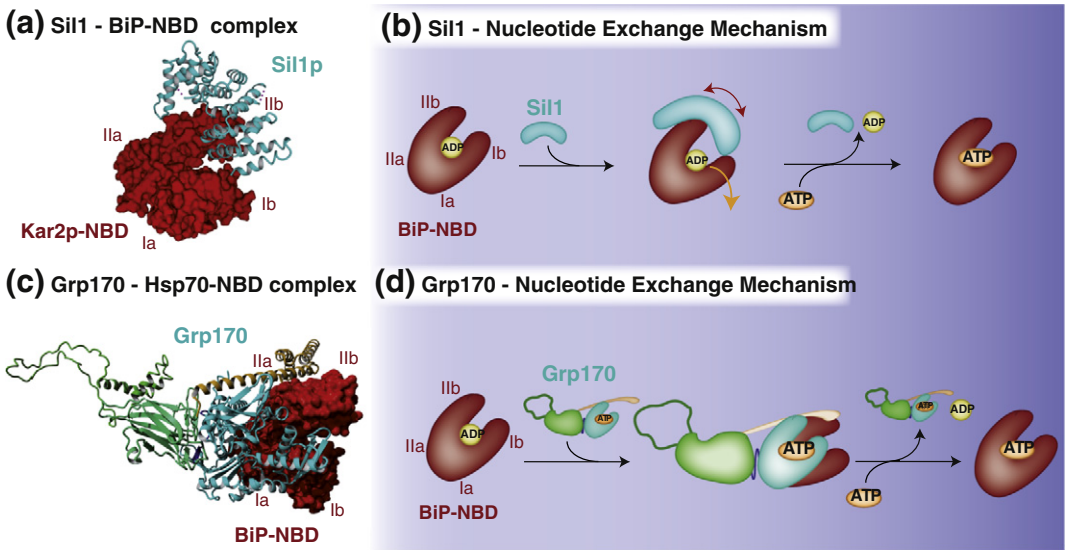

Fig. 4. Mechanisms used by Sil1 and Grp170 to regulate nucleotide exchange for BiP. (a) The crystal structure obtained for yeast Sil1 bound to the NBD of BiP (PDB ID: 3QML) [88] depicts how the single domain NEF Sil1, consisting of four armadillo-like repeats (cyan; shown in ribbon), wraps around lobe IIb of BiP's NBD (red; shown in surface representation) and displaces lobe Ib during the nucleotide exchange reaction. (b) Upon the binding of Sil1 to the ADP-bound form of BiP, the NBD cleft of BiP is opened by tilting lobe IIb and, to a lesser extent, lobe Ib outwards and thus destabilizing the domain and releasing ADP. ATP can subsequently bind to the NBD and BiP can re-enter its ATPase cycle. (c) The crystal structure of yeast Sse1p bound to the NBD of human Hsp70 (PDB ID: 3D2F) [112] was used to model Grp170 (NBD in cyan, β-sheet and unstructured loop in green, and α-helical domain in yellow; shown in ribbon) using Yasara Structure (www.yasara.org). Grp170 is shown bound to the human BiP NBD (PDB ID: 3LDL) [30] (red; shown in surface representation). Complex formation occurs *via* multiple contacts between the respective NBDs, and in addition, the C-terminal α-helical domain of Grp170 reaches out to embrace the Hsp70 NBD. (d) For the nucleotide exchange reaction to occur, Grp170 apparently binds to the ADP-bound form of BiP and destabilizes the structure of BiP's NBD resulting in the release of ADP. Once ATP is bound to BiP, the Grp170–BiP complex would dissociate and BiP can re-enter its ATPase cycle, although key steps in the NEF activity for large Hsp70 proteins remain to be elucidated.

Regulation, expression, and localization of Sil1

Although Sil1 appears to be present in the ER of all eukaryotic organisms examined, including *Arabidopsis* and rice plants, studies thus far have only been conducted in a few model organisms (Table 1). Even with this limited characterization, it is clear that species-specific differences exist. One of the first distinctions to be noted is its regulation by conditions that affect protein folding in the ER and activate the UPR, which generally regulates the expression of the majority of ER chaperones and their co-factors. For instance, in rice, when cells are treated with either dithiothreitol or tunicamycin, Sil1 is the most dramatically induced UPR target [89]. In *S. cerevisiae*, Sil1p (also known as PER100) was identified as a UPR-inducible gene [90], whereas, in *Y. lipolytica*, where Sil1 was first discovered, its induction by ER stress is only a very modest ~1.5-fold [91]. Lastly, in human cells, the *Sil1* gene does not appear to be a UPR target, and in fact, Sil1 protein levels may even be reduced in response to ER stress in cultured cells [5] or primary tissues [92]. Thus, Sil1 regulation by ER stress appears to run the gamut from super-induced to non-induced or possibly even repressed. The effect this might have on BiP-dependent folding or degradation during ER stress in these different organisms has not been directly addressed. There are only a few studies to examine the levels of Sil1 relative to BiP in various organisms or tissues, although a study using a human multitissue blot suggested that the mRNA levels of these two genes are coordinately expressed

Table 1. Species variations in Sil1 characteristics.

	UPR↑	ER retention	Role in translocation	Role in folding[a]
Y. lipolytica (Sls1p)	≤2-fold	+RDEL	+ +	+ +
S. cerevisiae (Sil1p)	+ + +	+RDEL	+	+/–
Human/mouse (BAP)	–	BiP association	ND[b]	+[c]
Zebra fish (Sil1)	+ +	?[d]	ND	ND

ND, not performed.
[a] As indicated by evidence of UPR activation in its absence.
[b] Not in plasma cells.
[c] Restricted to only certain tissues.
[d] Non-KDEL-like sequence (KMRQV) at C-terminus.

in a variety of tissues [5]. Secretory tissues, which have the highest levels of BiP, also express the greatest amounts of *Sil1* transcripts, and those tissues with lower levels of *BiP* have relatively reduced amounts of *Sil1*. A recent study reported that Sil1 protein was readily detected in mouse pancreas and, to a lesser extent, in liver but was undetectable in muscle [93], confirming that significant differences in Sil1 protein expression exist between various tissues. The relative quantities of BiP protein and a number of its co-factors were determined in canine pancreatic microsomes, where BiP was found to be expressed at a thousand times higher levels than Sil1 (5 μM *versus* 5 nM) [94]. The effects of ER stress on Sil1 expression in a human cell line suggest that the ratio of BiP to Sil1 would be even greater when ER homeostasis was disrupted [5].

Another significant difference among Sil1 proteins is the mechanism used to ensure that this soluble protein remains a resident of the ER. The majority of soluble ER chaperones and folding enzymes, like BiP and protein disulfide isomerase (PDI), possess a tetrapeptide sequence at their extreme C-terminus that is responsible for their retention in this organelle [95]. In the case of mammals, this is usually KDEL, whereas in birds, it is often RDEL, and in yeast, it is HDEL. This 4-amino-acid tag is recognized by a KDEL receptor that is located in the ER-Golgi intermediate compartment and the *cis*-Golgi. If a resident ER protein escapes the ER, it is caught by this receptor and returned to the ER lumen [96]. Indeed, inspection of the Sil1 homologues in *S. cerevisiae*, *Y. lipolytica*, several fungi, and mosquitoes reveals the presence of the R/K/DEL tetrapeptide at the C-terminus. However, in most metazoans and plants examined, there is not a readily recognizable KDEL-like sequence at the C-terminus of their Sil1 homologue. Instead, a significant number of them, including *Caenorhabditis elegans*, end with the sequence K/RELR, whereas in others, like *Xenopus laevis* and *Arabidopsis thaliana*, Sil1 possesses even more divergent C-terminal sequences. More recent studies have suggested that the KDEL receptor can recognize a large number of variations on this sequence [97], and thus, due to the highly conserved nature of the KELR tetrapeptide, it was assumed that it represented a divergent ER retention sequence [5]. Instead, it was recently revealed that this conserved sequence plays a critical role in structural integrity of the human Sil1 protein [98,99]. These residues are likely to form tertiary interactions with other portions of the protein that serve to stabilize an otherwise weak α-helix at the C-terminus [99]. The mechanism of ER retention for the human Sil1 protein was shown to occur through its interactions with BiP [99], which is present in much higher quantities than Sil1 [94] and possesses a KDEL sequence at its C-terminus as mentioned above.

Grp170: A NEF with a Chaperone Function

In addition to Sil1, a second NEF for BiP has been identified, Grp170. Based on similarities in domain organization, it was assigned to the family of large Hsp70s [100–102], which constitute the Hsp70 superfamily together with the conventional Hsp70s (Fig. 3). Although BiP and Grp170 share structural similarities, in contrast to BiP, where much is known about its various functions and how they are regulated [103], we are still lacking a clear understanding of Grp170's functions in the ER. Due to the similar domain organization, it was assumed that Grp170 was also a molecular chaperone that would interact with unfolded proteins in the ER in a similar manner as BiP. Therefore, the discovery that Lhs1p, the Grp170 homologue in yeast, could act as a NEF for yeast BiP, Kar2p [6], came as a surprise and immediately hinted toward a more complex function for this ER resident protein.

Regulation and structural organization of Grp170

Perturbations of ER homeostasis, by deprivation of either glucose in chick fibroblasts [104] or oxygen in human and rodent cell lines [105], resulted in the induction of a large-molecular-weight protein thus named Grp170 or ORP150 (*o*xygen-*r*egulated *p*rotein of *150* kDa), respectively. These were ultimately found to be the same protein, which will subsequently be referred to as Grp170. Soon after, related proteins were identified in *C. elegans* [100] and *S. cerevisiae*, Lhs1p [106–108], demonstrating that Grp170 was conserved in eukaryotes. Like BiP, the canonical ER-localized Hsp70 chaperone, Grp170 contains an ER targeting signal sequence, an N-terminal NBD, which binds to ATP even more efficiently than BiP [109], followed by a β-sheet domain, an α-helical domain, and a KNDEL ER retention sequence at its C-terminus [100,110]. Even though the NBDs between BiP and Grp170 are well conserved, their C-terminal regions are quite different. Unlike BiP, Grp170 possesses an acidic unstructured loop insertion in its β-sheet domain and a significantly extended and unstructured region at its C-terminus that in part accounts for its much larger size than BiP. Furthermore, inspection of the putative C-terminal SBD of Grp170 reveals significant differences compared to the corresponding region in BiP. This region in BiP forms an α-helical lid that stabilizes substrate binding to the β-sheet portion of the C-terminal SBD. However, structural studies on other cytoplasmic large Hsp70s [111–113] suggest that the α-helices comprising this region are too extended to allow the necessary kink to be formed so that this domain could serve as a lid. Instead, the extended α-helical domain in the

large Hsp70s was shown to reach out to embrace lobe II of the NBD of Hsp70 in a co-crystal structure to presumably facilitate the nucleotide exchange reaction [112,113] (Fig. 4c). Nevertheless, the recent structures of Hsp70s in their ATP-bound state [51,52] resemble the crystal structures of large Hsp70s [111–113]; as in both cases, the α-helical domain is extended and docks onto a NBD. No structural data on the conformation of the α-helical domain of large Hsp70s when they are in the ADP-bound state or bound to clients are yet available. In addition to the structural differences between canonical and large Hsp70s, there are a number of other distinctions. Unlike BiP, which is an unglycosylated chaperone, Grp170 has nine predicted glycosylation sites throughout the protein, with the majority of them mapping to the C-terminal region. Furthermore, a highly conserved Arg residue present in the NBD of all known canonical Hsp70s, which is critical for interaction with DnaJ family members [42], is missing in all ER-localized large Hsp70 family members that have been identified. Interestingly, this Arg is present in all the cytosolic orthologues, suggesting that the functional regulation of the ER proteins might be distinct from other canonical and large Hsp70-type proteins.

A chaperone function of Grp170

In spite of the fact that the more C-terminal regions of Grp170 are quite different from the SBD of BiP, Grp170 was first found in a complex with Ig heavy chains [114] and subsequently shown to bind thyroglobulin upon ER stress [115], as well as the non-secreted α1-antitrypsin Z mutant [116] and clusterin [117]. As all of these proteins also interact with BiP and because Grp170 interacts with BiP as its NEF [114,118], it was not clear if the binding of Grp170 to these substrates was direct or occurred indirectly *via* its association with BiP. In contrast to full-length proteins, radiolabeled peptides that were translocated into microsomes *via* the TAP transporter were shown to bind to Grp170 directly [119,120]. Further support for Grp170's chaperone function came from a study with purified proteins, where murine Grp170 was significantly more efficient in preventing the aggregation of heat-denatured luciferase than Hsc70 [121]. Similar data were obtained with yeast Lhs1p [122]. However, refolding studies conducted with murine Grp170 revealed that it failed to restore enzymatic activity in denatured luciferase in the presence of ATP and the cytosolic DnaJ family protein, Hdj-1. Only when rabbit reticulocyte lysate was added to the reaction could the activity of luciferase be recovered in an ATP-dependent manner [121], suggesting that either some of Grp170's functions depend on as yet-unidentified co-factors or that Grp170 maintains the denatured luciferase in a folding-competent state, which can then be folded by another chaperone. Solving the ambiguity whether Grp170 can bind clients in the absence of BiP, a recent study demonstrated that Grp170 continues to bind incompletely folded protein substrates after BiP is released with ATP, arguing for direct client binding [123]. As opposed to the nucleotide-dependent substrate binding of BiP, the interaction of Grp170 with its substrates is instead modulated by domains unique to large Hsp70s [123], suggesting a different regulation of substrate interaction for large Hsp70s compared to conventional Hsp70s. These insights beg the question of how substrates are released from the large Hsp70s. The first data on the biological functions of Grp170 came from a study conducted in both yeast and human cells, which revealed that degradation of free α-subunits of the epithelial sodium channel was dependent on Grp170's chaperone function but did not require its NEF activity [124] or even BiP [125]. Whether Grp170 and BiP together with an ERdj protein also form a protein disaggregation machinery, as recently shown for the cytosolic large Hsp70 orthologues [126,127], remains to be shown.

A number of recent studies have capitalized on Grp170's ability to bind peptides. Grp170 is being used to present antigens in various vaccine protocols [128–134], and the ability of a secreted form of peptide-bound Grp170 to stimulate antitumor immunity in cancer treatment has been tested [135–138] (reviewed in Ref.[139]). Two peptides that bound to the yeast cytosolic large Hsp70 Sse1p, but only poorly to the conventional Hsp70s Ssa1p or DnaK, were recently identified, whereas peptides that bound better to the conventional Hsp70s had either dramatically reduced or no binding to Sse1p, which suggested major differences in their binding specificities [140,141]. One of these peptides was shown to also bind Sse2p and human Hsp110, and mutational analysis of this peptide revealed that all three chaperones showed a preference for peptides containing aromatic residues [140,141]. Notably, the other peptide identified to bind Sse1 was also somewhat enriched in aromatic residues [140,141], although their contribution to Sse1 binding was not explored. This is in contrast to BiP, which prefers peptides with alternating hydrophobic residues [58,142], suggesting that conventional Hsp70s and large Hsp70s might each have some unique client proteins or that the two Hsp70 classes bind to different regions within a substrate.

Grp170 possesses nucleotide exchange activity

The first indications that Grp170 might possess nucleotide exchange activity for BiP were provided by a yeast genetic screen for suppressors of the severe growth defect observed in the *Δire1 Δlhs1* double mutant, which identified Sil1p [83]. Interestingly, BiP was unable to suppress this phenotype,

suggesting that Lhs1p might be functioning in this screen as something other than a molecular chaperone. The fact that yeast deficient in either Lhs1p or Sil1p was viable, while the *Δlhs1Δsil1* double mutation was lethal [83], led these investigators to suggest a common function for these two proteins. A few years later, Lhs1p was shown to serve as a NEF for Kar2p [6], and shortly thereafter, NEF activity was established for both *Y. lipolytica* [80] and mammalian [5] Sil1, demonstrating that the common function shared by these proteins was exchange activity. In subsequent years, NEF activity was detected for purified canine Grp170 [143], as well as other cytosolic large Hsp70s from a variety of organisms [144–149], thus revealing a completely new and unexpected function for these large Hsp70 proteins.

Insights into the mechanism by which the large Hsp70s stimulate nucleotide exchange were first obtained in studies with yeast where Lhs1p and Sil1p were found to bind to Kar2p in a mutually exclusive manner [6,143]. The interaction of Lhs1p with Kar2p is dependent on the presence of a nucleotide in the NBD of Lhs1p, and its exchange activity is enhanced by the addition of ER-localized DnaJ family proteins to stimulate ATP hydrolysis by Kar2p [122]. Crystallographic studies on Sse1p either alone [111] or bound to an Hsp70 NBD [112,113] revealed that the ATP-bound form of these large Hsp70s interacts directly with the NBD of Hsp70 in a head-to-head manner with multiple contacts occurring between domains IIa/b and Ia/b of Sse1p with domains Ia/b and IIb of the Hsp70 protein [104–106,111–113] (Fig. 4c). As noted above, additional interactions with the Hsp70 molecule occur through the α-helical bundle present at the C-terminus of the large Hsp70, which reaches over to embrace the Hsp70 NBD (Fig. 4c). The result of these interactions is to "pin" IIb lobe of the Hsp70 between Sse1's α-helical domain and its own subdomain Ib and to rotate Sse1's α-helical domain-sideways to release the nucleotide (Fig. 4d). Release of ADP from Hsp70 is not sufficient to trigger dissociation of the Hsp70NBD:Sse1p complex, arguing that rebinding of ATP to the Hsp70 NBD may be required [112,113]. This is in keeping with studies showing that Lhs1p can bind to Kar2p in either the apo form or the ADP-bound form but not the ATP-bound state [122].

Although a crystal structure for the ER-localized large Hsp70 bound to BiP has not been reported, based on the conformational dynamics of the Ssa1p NBD induced by Sse1p, the mechanism of the nucleotide exchange employed by Lhs1p is likely to be quite similar to that of Sse1p [150]. Hydrogen–deuterium exchange assays revealed that Lhs1p formed interactions with the NBD of Ssa1 that were very similar to those occurring with Sse1p, including the interaction of the α-helical bundle with the subdomain IIb. In addition, the residues that are critical for Sse1p's NEF function [112,113] are conserved in Lhs1p, and mutation of these residues similarly affected exchange activity in Lhs1p [150]. Together, these data make a strong case for the ER-localized large Hsp70s using the same mechanism of nucleotide exchange as has been established for their cytosolic orthologues.

Interestingly, despite the similarity in domain organization and the NBD structure between Grp170 and BiP, Sil1p does not physically interact with Lhs1p [6]. While both of these proteins promote nucleotide release from BiP, the binding of Lhs1p and Sil1p to Kar2p occur through distinct but mutually exclusive interactions [151], suggesting that they might compete for Kar2p binding. Studies to quantify levels of the two NEF proteins in the ER of canine pancreas revealed that Grp170 was present at much higher levels (~0.60 μM) than Sil1 (~0.005 μM) [94]. It is unclear if this is because Grp170 has additional functions or if the two proteins regulate nucleotide exchange to assist BiP in distinct activities or under unique cellular conditions. However, there are little data available to address either of two these possibilities. Clearly further studies aimed at dissecting how the two NEFs influence the functions of BiP *in vivo* are needed to understand the significance of the differences in their levels and how they assist BiP in its various functions.

Contribution of ER NEFs to Biological Functions

Cellular functions dependent on Sil1

While the ER functions that Sil1 participates in remain rather poorly understood, some insights have been obtained by examining the effects of gene disruption in organisms ranging from yeast to man. In humans, mutations in the *SIL1* gene have been found in over half the cases of Marinesco-Sjögren syndrome (MSS) [152–154], an autosomal recessive disease characterized by multisystem defects including cerebellar ataxia due to Purkinje cell loss, progressive myopathy, early-onset cataracts, skeletal anomalies, and a variety of developmental abnormalities and intellectual disabilities [155–158]. MSS-associated mutations occur throughout the *SIL1* gene and most lead to the disruption of significant portions of the protein [98,153,155], including those regions that interact with BiP. However, three MSS-associated mutations that disrupt only the last 4 or 5 amino acids of Sil1 have been identified, which affects its solubility and stability resulting in significantly diminished expression of these Sil1 mutants [98,99]. Five additional MSS-associated mutations that result in single-amino-acid substitutions in Sil1 have been

identified [98,154,155,157,159,160], although the effects of these mutations on Sil1 function or expression have not been examined. It remains unclear why only some tissues appear to be affected by mutations in the ubiquitously expressed Sil1 protein, and in most cases, the molecular mechanisms underlying the pathology remain unknown. Muscular hypotonia is often the initial symptom observed in children with MSS and presents early in infancy [161]. A number of electrophysiology [162] and ultrastructural studies [163–165] have been conducted on muscle biopsies from affected individuals, which reveal evidence of abnormal membrane function and structures, including a dilated sarcoplasmic reticulum, autophagic vacuoles, and the presence of electron-dense material suggestive of protein aggregates. A second common feature of MSS is ataxia due to severe atrophy of the cerebellum [166–168]. However, as most of these studies were performed before mutations in *SIL1* were linked to MSS, it is not clear if all of the affected individuals examined had *SIL1* mutations. To test the potential role of Sil1 in the cerebral cortex, mouse embryos were electroporated *in utero* with vectors encoding shRNA targeting *SIL1* [169]. Defects in neuronal migration were observed at birth in these mice, which were rescued by co-expression of human wild-type Sil1 but not by three MSS-associated Sil1 mutants.

Although a Sil1-null mouse is not available, the gene has been disrupted spontaneously by transposon insertion, *Sil1*^{wz}, and by gene-trap methodology, *Sil1*^{Gt}, resulting in the loss of amino acids 261–465 of the Sil1 protein [170]. Both genetic strains are referred to as woozy mice and have been reported to phenocopy some of the pathologies associated with MSS including cerebellar loss resulting in ataxia [170] and a progressive myopathy [164], which provide a more tractable model for understanding both the disease and function of Sil1. Inspection of quadriceps muscle tissue from woozy mice revealed structural changes that are similar to those reported in muscle biopsies from MSS patients. Western blot analyses of muscle lysates detected up-regulation of ER chaperones, including Grp170 and components of the ERAD, as well as the accumulation of ubiquitinated proteins and induction of autophagic responses compared to wild-type muscle lysates [164]. When the cerebella from these mice were examined, a progressive and profound Purkinje neuron degeneration was observed [170]. Electron micrographs and immunohistochemistry analyses revealed evidence of autophagosome-like structures, presence of ubiquitinated protein aggregates, and activation of ER stress and apoptotic responses in the Purkinje cells. Although Grp170 is modestly up-regulated in the Purkinje cells in the woozy mice, when they were crossed with transgenic mice overexpressing Grp170 (Tg-Hyou1), the resulting *Sil1*^{Gt}*;Tg-Hyou1* mice showed no obvious Purkinje cell loss or evidence of UPR activation in these cells, and the ataxia was dramatically suppressed [171]. Correspondingly, when the woozy mice were crossed with mice having decreased levels of Grp170 (Hyou1$^{+/-}$), Purkinje cell loss, ER stress, and ataxia occurred earlier and were more severe [171]. Interpretation of the results obtained with increased and diminished Grp170 expression are complicated by the fact that this large Hsp70 protein has both chaperone [121,123] and nucleotide exchange [143] activities. Thus, it is unclear whether Grp170 can provide the critical missing NEF function in Purkinje cells only when it is super-expressed or if it competes for binding to BiP clients under these overexpression conditions, thus alleviating the need for a NEF. As an alternative means of examining this point, the woozy mice were crossed with DNAJC3--null mice. DNAJC3/p58^{IPK}/ERdj6 is one of seven identified resident ER DnaJ family members [3,4] and can bind directly to some secretory pathway proteins and deliver them to BiP [172]. The resulting *Sil1*$^{-/-}$*;Dnajc3*$^{-/-}$ mice showed no signs of ataxia, Purkinje cell loss, or accumulation of protein inclusions. This suggests that the partial restoration of cerebellar function might be due to interfering with the targeting of a particular subset of clients to BiP, although what these clients are remains unknown. Most recently, studies using woozy mouse model demonstrated impaired ER homeostasis in motor neurons. When *Sil1* heterozygous mice were crossed with a mouse model of amyotrophic lateral sclerosis, Sil1 levels were progressively depleted in the fast-fatigable motor neurons leading to lowered excitability, a condition that was restored by Adeno-associated virus-mediated delivery of Sil1 to the motor neurons [173]. In fact, it is unclear whether the associated pathologies in this and other affected tissues arise from reduced maturation of essential secretory pathway proteins, depletion of free BiP stores resulting in activation of the ER stress response, toxicity due to aggregation of secretory pathway proteins, or even perhaps of the mutant Sil1 protein itself.

The woozy mice have also been the focus of recent studies on several additional organ systems. Sil1 is highly expressed in the β-islet cells of the pancreas of wild-type mice, and disruption of the *SIL1* gene resulted in decreased islet mass, evidence of UPR activation, and reduced plasma insulin levels upon glucose stimulation due to defects in insulin secretion [93]. This is in keeping with the fact that insulin is a BiP client [174,175] and might suggest that disruption of Sil1 reduces the secretion of insulin by inhibiting the release of BiP, although this was not directly shown. It is noteworthy that individuals with MSS show no evidence of defects in pancreatic function, demonstrating a clear difference between mice and man in the reliance of this

organ on Sil1 function. In a separate study using the woozy mice, the contribution of Sil1 to antibody assembly and secretion was investigated. BiP was originally identified in association with free Ig heavy chains in Abelson virus transformed pre-B cells [176], which are arguably the best-characterized BiP client. Although the co-expression of BiP mutants that cannot be released from clients by ATP inhibits both the assembly and the secretion of IgG antibodies [76], the Sil1^{Gt} mouse was indistinguishable from wild-type controls in terms of both the kinetics and the magnitude of antigen-specific antibody responses and in Ig assembly from LPS-stimulated splenic B cells [177]. This was also confirmed in three human EBV-transformed lymphoblastoid cell lines obtained from individuals with MSS, arguing that the BiP co-factor Sil1 is dispensable for antibody production in both of these species.

Biological functions requiring Grp170

A large variety of biological functions have been associated with Grp170, although molecular insights into the role of Grp170 in these processes are generally lacking (also reviewed in Ref.[178]). Loss or decreased expression of this ER-localized large Hsp70 protein inhibits the translocation of nascent polypeptide chains into the ER in a number of organisms as revealed by both genetic and biochemical experiments [106–109]. In *S. cerevisiae*, *lhs1*-null cells exhibit a selective translocation defect that affects a subset of proteins, including preKar2p, prePDI, prepro-α-factor, and preCPY, whereas the translocation of another SRP-dependent protein, dipeptidylaminopeptidase B was not affected [106–108]. Lhs1p's NEF activity was shown to be critical to support BiP-mediated protein translocation into the ER, but this activity was not required for its chaperone function [122,124]. When mammalian proteoliposomes were depleted of ATP-binding proteins, preprolactin import was inhibited. Translocation was restored when the microsomes were repleted with the ATP-binding proteins but not with purified BiP alone [109]. Since BiP and Grp170 were the major ATP-binding proteins identified in the microsomes, it is reasonable to conclude that Grp170 plays a critical role in the import of this protein. However, since BiP is also required for protein translocation into the yeast ER [179,180], it remains unclear whether Grp170 itself plays a direct role in protein translocation through its chaperone function or whether nucleotide exchange by Grp170 is a rate-limiting factor for BiP's role in protein translocation. A very recent study demonstrated, however, that the NEF function of Grp170 is critical for BiP release and retrotranslocation of the SV40 virus from the ER into the cytosol [181].

Grp170 has also been implicated in a variety of physiologically relevant protein secretion processes linked to disease states. The high expression of Grp170 in pancreatic islets is significantly reduced by fasting, suggesting that it may have a role in insulin biosynthesis [182]. The effects of both increased and decreased Grp170 expression levels on insulin production and blood glucose levels have also been examined using a number of normal mice and mouse models for type II diabetes. Increasing Grp170 levels with adenovirus-delivered constructs increased insulin secretion and decreased blood glucose after feeding, whereas decreasing Grp170 expression with antisense constructs inhibited insulin secretion and resulted in significantly higher blood glucose levels [183]. In another study, Grp170 levels were modulated by crossing the diabetes-prone Akita mice to either Grp170$^{(+/-)}$ heterozygous mice or Grp170 transgenic mice [184]. Decreased expression of Grp170 accelerated the onset of diabetes in this model, whereas transgene-enhanced expression of Grp170 resulted in lower body weight and improved glucose tolerance in young mice. Interestingly, there were no changes in the level of insulin in the pancreatic cells of either genetic model, which could suggest that the retained insulin was continuously being degraded in the animal model. It is noteworthy, as discussed above, that disruption of Sil1 also results in a similar pancreatic phenotype in mice [93], raising the question of why both proteins are required for insulin biosynthesis and secretion.

Similar to its effects on insulin secretion, a number of studies have indicated a role for Grp170 in VEGF (*v*ascular *e*ndothelial *g*rowth *f*actor) secretion, one of the most important proangiogenic factors. Decreased Grp170 levels in C6 glioma cells [185] and macrophages [186], achieved by expressing an antisense-Grp170 construct, resulted in an inability of both cell lines to secrete VEGF into culture media under hypoxic conditions. In the case of C6 cells, the intracellular levels of VEGF were dramatically increased, arguing for an inhibition of protein transport, possibly due to improper maturation when Grp170 levels were reduced. Conversely, when Grp170 was overexpressed, cells secreted significantly higher levels of VEGF compared to non-transfected cells [185,186]. Under hypoxic conditions, Grp170 could be co-immunoprecipitated with VEGF and *vice versa*, indicative of an interaction between these proteins [185,186]. The contribution of Grp170 to the expression of this proangiogenic factor may in part explain its contribution to tumor survival and metastasis [139].

Grp170 levels have also been associated with cytoprotection during other physiological and disease states. Ischemia and atherosclerotic plaque formation lead to lower oxygen levels in the surrounding tissue, which induces ER stress and hypoxic pathways, resulting in cell death if not resolved. Transgenic mice overexpressing Grp170 lost significantly less brain tissue in response to ischemia [187], and upon kainate

administration, fewer animals suffered from seizures compared to wild-type mice [188]. The survival rate of neurons after hypoxic stress [187,189] or after excitotoxicity upon glutamate administration [188,189] also increased when Grp170 was over-expressed. Regulated cell death is an important event in many developmental phases, including in the brain [190]. Somewhat counter-intuitively, Purkinje cells were shown to significantly up-regulate Grp170 expression during the peak phase of programmed cell death in mouse brain development. However, overexpression of Grp170 in the transgenic mouse model led to increased survival of these cells during development, resulting in a higher number of Purkinje cells compared to wildtype [191], suggesting possible alternative concentration-dependent effects of Grp170 over-expression. Of note, this abnormal brain development resulted in an impaired motor coordination of these mice. Under another pathological condition, the accumlation of atherosclerotic plaques, which deprive surrounding tissue of their oxygen supply, lead to up-regulation of hypoxic and ER stress pathways in segments of aortae with severe atherosclerosis [192–194]. In particular, macrophages, one of the key players in the pathogenesis of atherosclerosis within the atherosclerotic plaque [195], showed significant induction of stress pathways including up-regulation of Grp170 [192]. When Grp170 levels were reduced in cultured mononuclear phagocytes, the cells became more susceptible to hypoxic stress, which was exacerbated when this was combined with oxidized LDL [192]. Similarly, overexpression of Grp170 in a microvascular endothelial cell line prevented ER stress activation [194] and inhibited apoptotic cell death [193] triggered by oxLDLs. Hence, Grp170 promotes cell survival under a number of physiological stress conditions.

Concluding Thoughts

While the past decade has significantly increased our understanding of how the ATPase cycle of BiP is regulated, this information has led to many more questions. First, it is unclear what the relative contributions of Sil1 and Grp170 are to BiP's requirement for exchange activity, how exchange activity contributes to the various biological functions of BiP, and whether this differs in individual tissues or under various developmental or stress conditions. Mechanistically, it is not completely understood how these NEFs distinguish between the different nucleotide states of BiP. Is the ATP/ADP ratio in the ER together with nucleotide-induced structural changes in the NBD sufficient to control the specificity or do nucleotide-regulated changes in other regions of the BiP molecule also contribute? Second, although genetic complementation studies reveal that either NEF is able to compensate for loss of the other factor to varying degrees, based on studies conducted in organisms ranging from yeast to humans it is clear that these factors are not entirely interchangeable and that some defects remain upon complementation or that decidedly non-physiological levels of expression must be used to suppress phenotypes. This is underscored by the fact that the deletion of Grp170 is embryonic lethal in mice [188], whereas loss of Sil1 causes a multisyndrome disease in both humans [153] and mouse models [170]. Lastly, the finding that Grp170 possesses both chaperoning and NEF activity makes it more complicated to determine which activity is critical in a number of the functions for which it has been implicated and how substrate binding and NEF activity of Grp170 contribute to the folding environment of the ER.

Acknowledgements

J.B. gratefully acknowledges funding by the Boehringer Ingelheim Fonds; M.J.F., by the German Academy of Sciences Leopoldina Grant Number LPDS 2009-32; and L.M.H., by the National Institutes of Health Grant Number R01 GM054068.

Conflict of Interest Statement: The authors declare no competing financial interests.

Received 23 December 2014;
Received in revised form 10 February 2015;
Accepted 10 February 2015
Available online 16 February 2015

Keywords:
BiP;
Grp170;
Sil1;
protein folding;
nucleotide exchange factors

†J.B. and M.J.F. contributed equally to this work.

Abbreviations used:
BAP, BiP-associated protein; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERQC, ER quality control; MSS, Marinesco-Sjögren syndrome; NBD, nucleotide binding domain; NEF, nucleotide exchange factor; SBD, substrate binding domain; UPR, unfolded protein response.

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