Leading Edge
Review

Cell

Molecular Chaperones and Protein Quality Control

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In living cells, both newly made and preexisting polypeptide chains are at constant risk for misfolding and aggregation. In accordance with the wide diversity of misfolded forms, elaborate quality-control strategies have evolved to counter these inevitable mishaps. Recent reports describe the removal of aggregates from the cytosol; reveal mechanisms for protein quality control in the endoplasmic reticulum; and provide new insight into two classes of molecular chaperones, the Hsp70 system and the AAA+ (Hsp100) unfoldases.

Protein Folding in the Endoplasmic Reticulum: Knowing Right from Wrong

The endoplasmic reticulum (ER) is responsible for the structural maturation of the roughly one-quarter of the proteome that traverses the secretory pathway (Anken et al., 2005). Folding of secretory proteins provides a number of unique challenges. Folding is often accompanied by and dependent on the formation of correct native disulfide bonds and insertion into the lipid bilayer, with both events occurring more slowly by orders of magnitude than the typical conformational changes that accompany folding. Correspondingly, the ER provides an environment optimized to face these challenges, including high concentrations of general chaperones as well as a range of strategies specifically tailored to aid folding of secretory proteins. Additionally, a sophisticated quality-control system exists in the ER to retain and retrieve proteins that have not yet reached their native state (Ellgaard and Helenius, 2003).

Despite the lengths to which the cell goes to provide an optimized environment in the ER, folding of secretory proteins can and does fail, at times at an alarming rate. The ER employs two distinct mechanisms for responding to the presence of misfolded forms. The first is an ER-dedicated stress response termed the unfolded protein response (UPR), which acts to remodel the ER so as to increase its folding capacity (Schroder and Kaufman, 2005). The second, termed ER-associated degradation (ERAD), specifically recognizes terminally misfolded proteins and retrotranslocates them across the ER membrane into the cytosol, where they can be degraded by the ubiquitin-proteasome degradation machinery (Romisch, 2005). These two systems are intimately linked: UPR induction increases ERAD capacity, loss of ERAD leads to constitutive UPR induction, and simultaneous loss of ERAD and the UPR greatly decreases cell viability. The UPR and ERAD systems also face the common problem of recognition, which requires the identification of potentially pathological misfolded forms in an ER environment that is constitutively filled with normal on-pathway folding intermediates. UPR and ERAD surveillance must strike a fine balance, protecting the cell from dangerous misfolded species while avoiding overvigilance (as happens with mutant forms of the cystic fibrosis transmembrane regulator [CFTR]), which can lead to the disposal of potentially remediable forms. Recent advances reveal how the UPR and ERAD identify misfolded forms.

The Unfolded Protein Response

In yeast, the folding capacity of the ER is monitored by IRE1, a highly conserved transmembrane kinase that contains a lumenal domain responsible for sensing misfolded forms and cytosolic kinase and ribonuclease domains. The accumulation of misfolded proteins in the ER leads to activation of the IRE kinase. IRE kinase activation promotes the nonconventional splicing of the message for a b-ZIP transcription factor (Hac1p in yeast and XBP-1 in metazoans) via its ribonuclease domain. Translation of the spliced Hac1 message creates an active transcription factor that directly mediates transcription of UPR targets including ER chaperones, the ERAD machinery, and a range of other secretory proteins.

In addition to IRE1, higher eukaryotes utilize two other sensors, the ER transmembrane kinase PERK and the ER transmembrane transcription factor ATF6. PERK contains a lumenal sensor that is highly related to that of IRE1, but, unlike IRE1, the PERK cytoplasmic domain consists of an eIF2α kinase. Activation of the kinase by the presence of misfolded proteins results in a general-

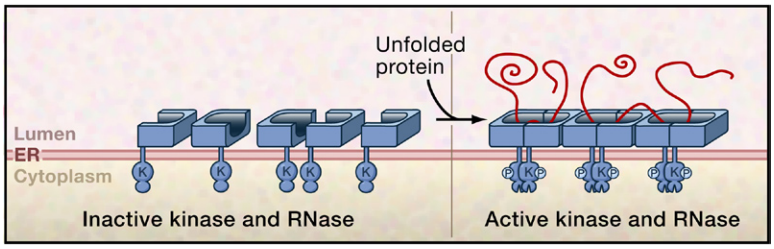

Figure 1. Unfolded Protein Recognition by Ire1

A hypothetical model depicts Ire1 activation through oligomerization brought about by binding of unfolded proteins (indicated in red). Direct or indirect interactions between unfolded protein chains may contribute to activation. On the lumenal side of the ER membrane, the postulated unfolded protein binding groove formed by the dimerization of the Ire1 core lumenal domain through interface 1 is indicated in gray. On the cytoplasmic side of the ER membrane, oligomerization juxtaposes the Ire1 kinase domains, which undergo a conformational change following autophosphorylation leading to activation of the RNase function of Ire1. Inactive Ire1 could be monomeric as shown or could already exist in oligomeric yet inactive states whose quaternary associations change upon the binding of unfolded protein. Figure modified from Credle et al. (2005).

ized inhibition of translation as well as the upregulation of a specific transcription factor, ATF4. Accumulation of misfolded proteins also allows ATF6 to reach the Golgi, where transmembrane proteases release the cytoplasmic transcription-factor domain, allowing it to enter the nucleus and mediate gene induction. The existence of multiple UPR sensors in higher eukaryotes allows for a more nuanced response to misfolded proteins. For example, an initial response to protein misfolding could be a generalized downregulation of translation, followed in sequence by induction of chaperones; the induction of the ERAD machinery; and ultimately, in the face of prolonged stress, activation of cell death via apoptosis.

Very recently there have been exciting advances in our molecular understanding of how IRE1 (and, by inference, PERK) recognizes misfolded proteins. Earlier studies observed that the major ER-localized Hsp70 homolog BiP specifically binds IRE1 and that this interaction disappears under conditions of ER stress. Additionally, overexpression of BiP can suppress the induction of the UPR. This had suggested a titration model, in which BiP acts as a negative regulator of IRE1 and the accumulation of misfolded forms leads indirectly to IRE1 activation by the sequestration of BiP by misfolded proteins. However, recent studies indicate that although BiP binding is likely to play an important role in down regulating IRE1, it is not the whole story. Mutational analysis found that deletion of the region of IRE1 responsible for BiP binding did not impair the regulation of IRE1 in the response to unfolded protein (Kimata et al., 2004). More dramatically, the crystal structure of the conserved core lumenal domain (cLD) of yeast IRE1 reveals a deep hydrophobic groove reminiscent of the binding pocket in the major histocompatibility complexes (MHCs) that is responsible for peptide recognition. That IRE1 may directly bind misfolded polypeptides is an appealing idea (Figure 1) (Credle et al., 2005). By directly recognizing misfolded forms, the initiation of IRE1 induction could occur prior to the full titration of BiP, which, given BiP's extremely high abundance in the ER, might occur only after a catastrophic accumulation of misfolded proteins. Additionally, direct recognition of misfolded forms by IRE1 (and PERK) could allow for a more nuanced set of responses in which different misfolded forms could be preferentially recognized by the different sensors (BiP, IRE1, or PERK). Thus, in principle, the nature (e.g., translational versus transcriptional) and timing of the UPR could be tailored to the specific class of misfolded forms that are prevalent in the ER.

Endoplasmic Reticulum-Associated Degradation

As might be expected by the diversity of proteins that fold in the ER, recent studies argue that ER-associated degradation (ERAD) encompasses a number of different systems, each responsible for the degradation of subsets of proteins that share common physical properties. This is perhaps most clearly shown in yeast, where there are at least two distinct surveillance mechanisms for identifying terminally misfolded ER proteins. The first, designated ERAD-L, inspects for proteins that contain misfolded lumenal (soluble or membrane-tethered) domains such as CPY*, a mutant form of the endogenous CPY protein that is incapable of folding. The second, termed ERAD-C, detects misfolded cytosolic domains of transmembrane proteins (Vashist and Ng, 2004). Although both of these pathways ultimately converge on the ubiquitin-proteasome degradation system, they depend on different sets of ER-associated components to detect and deliver misfolded species to the cytosol. In the case of ERAD-C (but not ERAD-L), degradation is typically dependent on a specific subset of cytosolic chaperones including Hsp70 and Hsp40 members (Nishikawa et al., 2005). This suggests that these proteins may be directly responsible for recognizing misfolded cytosolic domains of transmembrane proteins. However, the exact features that are being monitored and how substrates are delivered to the retrotranslocation machinery remain important open questions for most substrates.

The recognition of a protein containing a misfolded lumenal domain is critically dependent on the protein's glycosylation status (Helenius and Aebi, 2004). Indeed, even minor alterations of N-linked glycans can lead to severe defects in the degradation of a number of substrates. This can effectively leave proteins in limbo: Unable to reach the native state but not recognized by the ERAD machinery, they remain misfolded in the ER indefinitely. At first blush, this reliance on glycosylation appears to be an unneeded embellishment. However, it

is now clear that the spectrum of sugar moieties present on a protein is a key signal in marking its folding status. Indeed, given that several highly abundant proteins resident in the ER, including BiP and PDI, are often not glycosylated, the presence and covalent nature of high-mannose sugars on a polypeptide may provide an important signal that helps the ER quality-control machinery to distinguish folding species destined for other compartments from the more abundant permanent residents of the ER. Insight into the role of glycosylation in the discrimination between on-pathway folding species and terminally misfolded proteins in the ER has come from the finding that misfolded glycoproteins undergo trimming of their N-glycans by a variety of glycosidases including a specific mannosidase. In conjunction with persistent protein misfolding, the time-dependent remodeling of N-glycans is thought to result in a bipartite signal for degradation by the ERAD machinery. This affords folding intermediates a period of time, before the remodeling of their N-glycans, in which the polypeptide is immune from surveillance by the ERAD machinery. Interestingly, a recent study suggests that even for cytosolic polypeptides, there is a period during and shortly after synthesis in which misfolded species are immune from degradation by the proteasome machinery (Vabulas and Hartl, 2005).

Recent studies have identified two different ER-localized lectins that play a critical role in ERAD. The first is related to the mannosidase protein responsible for the trimming of N-glycans in the ER but appears to have lost its catalytic activity. In yeast it is called Htm1p/Mnl1p, and in mammals it is called EDEM (for ER degradation-enhancing α-mannosidase-like protein). Studies in mammalian cells suggest that EDEM helps misfolded glycoproteins leave the calnexin/calreticulin lectin chaperone cycle, where they are attempting to fold, and enter the degradation pathway (Molinari et al., 2003; Oda et al., 2003). Nonetheless, in yeast, Htm1p is required for efficient degradation of substrates such as CPY* that do not depend on calnexin. Thus, it seems likely that Htm1p/EDEM plays other roles in ERAD. The second lectin, Yos9p, forms a stable complex with misfolded proteins, and loss of Yos9p leads to a profound and specific defect in degradation of misfolded glycoproteins (Cormier et al., 2005). Surprisingly, whereas point mutations in the Yos9p mannose binding pocket eliminate its ability to support ERAD, the same Yos9p mutants show enhanced interactions with substrates. This raises the intriguing (albeit speculative) possibility that the Yos9p lectin is playing a more informational role, querying the sugar status of misfolded forms to determine whether they should be passed on to the retrotranslocation machinery. However, as with ERAD-C, although there has been dramatic progress in characterizing the retrotranslocation machinery (Romisch 2005, 2006), which structural features in misfolded proteins are being monitored and how the recognized misfolded forms are handed off to this machinery remain poorly understood.

Aggregate Clearance via Autophagy

It has become increasingly apparent that there are a variety of conditions in vivo where, even with chaperones and the proteolytic machinery present in the same compartment as a misfolding protein, these mechanisms of quality control fail and the misfolded proteins proceed to form aggregates. Moreover, such intracellular aggregates are associated with a number of neurodegenerative diseases such as Huntington's and Parkinson's. The nature and fate of protein aggregates in eukaryotic cells has been poorly understood. Most recently, a protective action of aggregate formation, as opposed to an immediately pathogenic role, has been increasingly supported. For example, in the case of Huntington's disease, serial examination of neuronal cells in culture overproducing a polyglutamine-expanded huntingtin-GFP fusion revealed that those cells containing morphologically visible fluorescent aggregates exhibited better viability than those bearing diffusely fluorescent material (Arrasate et al., 2004). This seems consistent with the concept that it is small assemblies of misfolded proteins, not morphologically visible inclusions, that exert toxic effects on cells. Correspondingly, a recent comparison of size versus toxicity for aggregates of the prion protein PrP suggested that small aggregates containing one to two dozen molecules were the most toxic to cells (Silveira et al., 2005). But do cells have mechanisms for clearing these small aggregates—or, for that matter, larger ones?

Earlier studies suggested that the ubiquitin-proteasome pathway might be the mainstay of removal of aggregation-prone species. Indeed, aggregates detected in the setting of neurodegeneration are usually reactive with anti-ubiquitin antibodies, implying that the misfolding species have been recognized by the ubiquitin conjugation system. Yet evidence of the last few years indicates that these modified proteins present a particular challenge to proteasomes, possibly leading to their inhibition (Bence et al., 2001; Bennett et al., 2005). The mechanism of this inactivation remains unknown but may involve the "choking" of the proteasome chamber (Venkatraman et al., 2004).

It appears now that ubiquitin modification may in fact recruit aggregated species for clearance via an independent mechanism, the "autophagy" pathway. Autophagy involves the recognition and packaging/engulfment of targeted proteins or organelles into autophagosome vesicles that become fused with lysosomes, wherein both vesicles and their contents are broken down (Levine and Klionsky, 2004). More than 20 so-called Atg components mediate this remarkable process. Recent experiments knocking out central components of autophagosome formation in mice have begun to demonstrate its scope of activity in mammalian tissues. For example, deficiency of atg5 led to death of newborn animals due to inability to provide a supply of amino acids via degradation of body protein during the relative starvation conditions of the immediate postnatal period (Kuma et al., 2004).

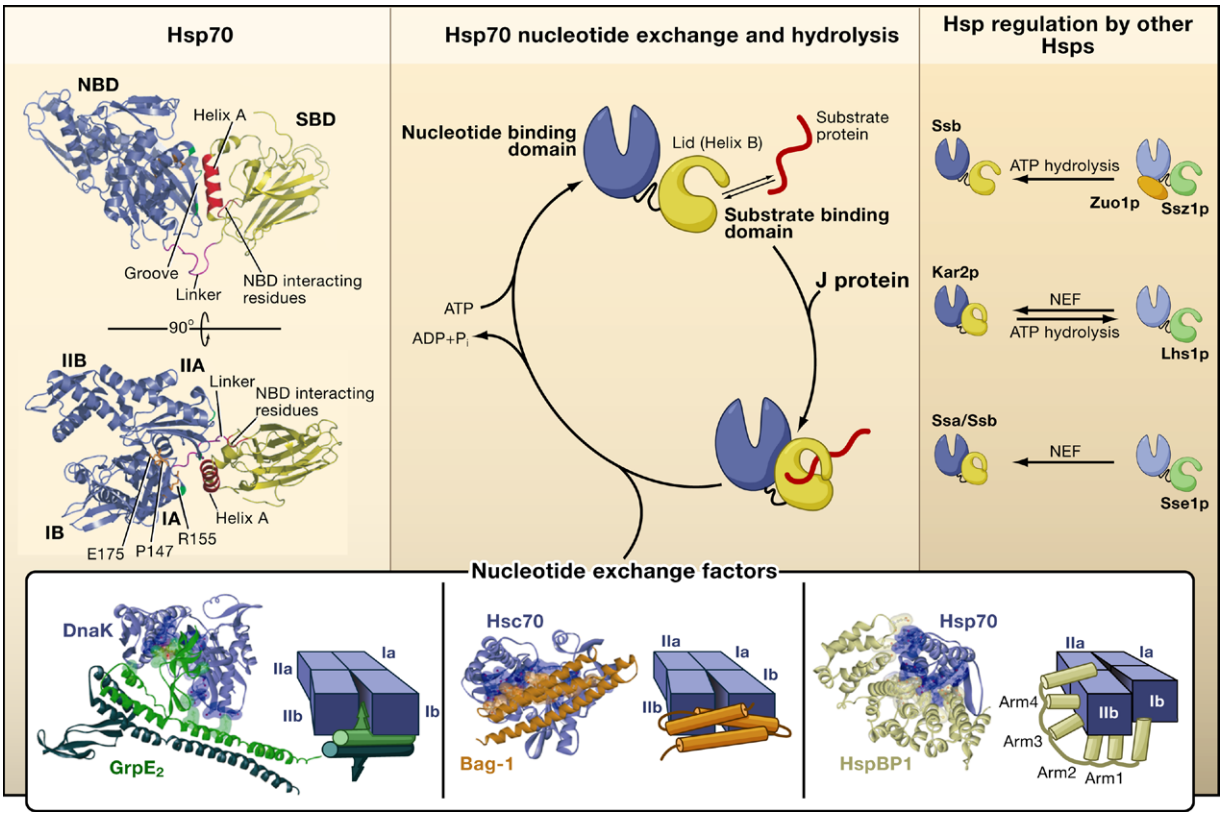

Figure 2. Hsp70 Interactions

Left panel: Communication between the nucleotide binding domain (NBD, blue) and the substrate binding domain (SBD, yellow) of an Hsp70, as indicated from the crystal structure of bovine Hsc70 (Jiang et al., 2005), featuring a 10 residue linker segment (purple) and an interaction between helix A, a few residues of the β sandwich in the SBD (both shown in red), and a groove in the NBD (green). Signal transduction from the catalytic center of the NBD to the interdomain interface is mediated by a hydrogen bond network with key residues (orange) being E175 as nucleotide sensor, P147 as structural switch, and R155 as surface-exposed relay (Vogel et al., 2006).

Middle panel: The features of the Hsp70 cycle. NBD is shown in blue, SBD in yellow, and substrate protein in red. J proteins stimulate ATP hydrolysis, locking substrate into the SBD; NEF proteins exchange ATP for ADP, leading to substrate release.

Right panel: The effects of Ssz1p, Lhs1p, and Sse1p on the Hsp70 cycle of three specific Hsp70 proteins, Ssb, Kar2p, and Ssa/Ssb, are indicated with arrows. e.g., Ssz1p in complex with zuotin (Zuo1p) stimulates ATP hydrolysis of Ssb (see text for additional detail).

Inset panel: Interactions of nucleotide exchange factors (NEFs) with Hsp70 nucleotide binding domains are revealed by three cocrystal structures, showing a number of different ways in which NEF proteins contact the ATP binding domain to open up its cleft to enable nucleotide exchange. Ribbon diagrams of these structures are shown for DnaK-GrpE2 (Harrison et al., 1997), Hsc70-Bag-1 (Sondermann et al., 2001), and Hsp70-HspBP1 (Shomura et al., 2005). At the right side of each ribbon diagram is a schematic showing how contacts between the NEFs and the NBDs serve to open the cleft between subdomains to enable nucleotide exchange.

Strikingly, in another study, in which atg7 deficiency was induced later in life using a "FLOXed" gene, accumulation of ubiquitin-positive aggregates was observed in the livers of animals that were disabled in the production of autophagosomes (Komatsu et al., 2005). Proteasome function in such animals was unaffected, arguing that aggregates, containing misfolded ubiquitin-tagged species, may normally be removed by the autophagy pathway. Further insight into such a recruitment mechanism comes from studies in cultured cells expressing expanded GFP-huntingtin, which observed a component known as p62 (or sequestosome1) forming a "shell" around the huntingtin aggregates (Bjørkøy et al., 2005). This protein contains a C-terminal ubiquitin-associated (UBA) domain that can bind polyubiquitin, such that p62 colocalized with ubiquitin when expressed in HeLa cells. Moreover, in the huntingtin-expressing cells, p62 colocalized with LC3, a protein that becomes localized to autophagosomes. Consistent with both reports showing that autophagy reduces levels of huntingtin aggregates (Ravikumar et al., 2004; Iwata et al., 2005) and with a critical role of p62 in this process, antisense-mediated inhibition of p62 expression increased apoptosis of the huntingtin-expressing cells. Thus, it seems that ubiquitin modification of defective proteins may provide an entryway to either the proteasomal system or, in contexts where aggregation is occurring, the autophagy system.

Hsp70 Chaperone Systems

70 kDa heat-shock proteins (Hsp70s) are engaged in a plethora of folding processes including the folding of newly synthesized proteins, the transport of proteins

across membranes, the refolding of misfolded and aggregated proteins, and the control of activity of regulatory proteins. This versatility is achieved through the evolutionary amplification and diversification of *hsp70* genes, which has generated both specialized Hsp70 chaperones and more diverged Hsp110 and Hsp170 proteins. Versatility is also achieved through extensive employment of cochaperones, J proteins, and nucleotide exchange factors (NEFs), which regulate Hsp70 activity (see Figure 2). Recent studies have advanced our knowledge of the Hsp70 machine and its interactions with its cochaperones. Surprisingly, these studies have also uncovered functional liaisons between Hsp70s themselves.

Allosteric Crosstalk in Hsp70

Hsp70s transiently associate with hydrophobic peptide stretches exposed in client proteins via a substrate binding domain (SBD) (Figure 2), thereby preventing aggregation and promoting proper folding. ATP binding to the N-terminal nucleotide binding domain (NBD) induces conformational changes in the adjacent SBD, which opens the substrate binding pocket and its helical lid (Figure 2). Conversely, substrate binding in synergy with the action of J proteins triggers ATP hydrolysis and concomitant closing of the SDB, which traps substrate proteins. Until recently, atomic structures were available only for the individual domains of Hsp70, which precluded a mechanistic understanding of interdomain communication. Sousa and coworkers have now solved the structure of bovine Hsc70 in a nucleotide-free state at 2.6 Å resolution. The structure (Figure 2) lacks only 10 kDa from the C terminus, leaving the substrate binding pocket intact (except the distal end of its helical lid) (Jiang et al., 2005). It had been shown from previously solved structures of isolated domains that the NBD consists of an actin-like fold with two globular subdomains separated by a nucleotide binding cleft whereas the SBD has a β sandwich that forms the substrate binding pocket, with an α-helix packed against the sandwich from one side (helix A) and a helical lid (helix B) closing on top of the substrate binding pocket. The new structure now reveals the elements providing the interdomain interaction, involving a flexible linker of 10 residues that connects the NBD and SBD; helix A of the SBD is embedded into a groove at the base of the NBD, and an additional contact is made between a few residues of the β sandwich of the SBD and the NBD groove (Figure 2). ATP binding may rearrange the interface between the NBD groove and SBD helix A, perhaps even disrupting it, thereby facilitating the opening of the SBD. Concomitantly, the linker may relocate and become more tightly associated with the connecting region, such that at no stage during the functional cycle of Hsp70 do the SBD and NBD become completely disconnected. It appears that the additional minor contact involving residues of the β sandwich of the SBD plays a critical role because a mutant with alteration in one of the involved residues has coupling defects (Montgomery et al., 1999). Signal transduction between SBD and NBD thus seems to rely on integrated rearrangements of the linker and at least two contact sites within the SBD.

Progress has also been made concerning the mechanism by which the structural changes resulting from ATP binding and hydrolysis are transmitted within the NBD. A hydrogen bond network relays the conformational signal within the NBD of bacterial DnaK, starting at a glutamate residue in the catalytic center and converging onto a surface-exposed, universally conserved arginine that is part of the region forming the interdomain interface (Vogel et al., 2006). At a central position within this network is a highly conserved proline residue that appears to establish the high energy barrier between the ADP and ATP bound states of DnaK, thereby constituting a molecular switch that uses the surface-exposed arginine as a relay to control the opening and closing of the SBD. An attractive speculation is that the switch operates through a *cis-trans* isomerization of the prolyl peptide bond.

Hsp70s—Nucleotide Exchange Factor Diversity

Several new findings provide insights into the intriguing mechanistic diversity by which cofactors regulate Hsp70 machines. NEFs are critical for the functional cycle of Hsp70s because they promote the release of ADP and rebinding of ATP that triggers unloading of bound substrate. Well-known NEFs are GrpE, which facilitates nucleotide dissociation from DnaK, and the heterogeneous family of BAG proteins, which are NEFs for cytosolic Hsp70s in eukaryotes. NEF activity has also been demonstrated for members of an abundant third family of proteins with poorly understood cellular functions, including the cytosolic Fes1p of *S. cerevisiae*; its human ortholog Hsp70 binding protein 1 (HspBP1); and the lumenal ER protein of *S. cerevisiae*, Sls1p.

The availability of cocrystal structures for three NEFs—GrpE (Harrison et al., 1997), Bag-1 (Sondermann et al., 2001), and now HspBP1 (Shomura et al., 2005)—in complex with the NBD fragments of their partner Hsp70s provides an impressive demonstration of the mechanistic diversity of the exchange reaction. The three NEFs not only are structurally unrelated but employ different modes of action (Figure 2). Although GrpE and Bag-1 both induce a 14° outward rotation of one of the NBD subdomains (IIB) that disrupts the nucleotide binding site, the two proteins use different strategies. GrpE binds to the back side of the NBD (in the view shown in Figure 2) and reaches deep into the nucleotide binding cleft, thereby forcing the opening of subdomain IIB. In contrast, BAG-1 contacts both sides of the central cleft from the top, thereby forcing the opening of IIB. The structure of the complex between HspBP1 and the Hsc70 NBD shows that this NEF consists of four α-helical armadillo-like folds. The armadillo-like folds form a curved structure (Shomura et al., 2005) that wraps around subdomain IIB from the side, presumably resulting in a steric clash with subdomain IB. HspBP1 may induce a distortion of the two lobes of the NBD relative to each other that disrupts

the nucleotide binding pocket. These structures are a beautiful demonstration of functional convergence of unrelated proteins to catalyze the nucleotide exchange reaction by Hsp70.

Hsp70-Hsp70 Liaisons

Intriguingly, the Stirling lab observed that a member of the Hsp170 family can act as a NEF for an Hsp70 (Steel et al., 2004). In particular, Lhs1p, the Hsp170 of the ER lumen of *S. cerevisiae*, acts as a NEF for the lumenal Hsp70, Kar2p. Conversely, Kar2p stimulates ATP hydrolysis of Lhs1p (Figure 2). The functional consequence of this reciprocal activation remains elusive. A further Hsp70-Hsp70 liaison has also recently been described in the yeast cytosol between the Hsp110 chaperone, Sse1p, and either Ssa or the ribosome-associating Ssb (Shaner et al., 2005; Yam et al., 2005), which are both cytosolic Hsp70 family members. The heterodimeric Sse1p-Ssa and Sse1p-Ssb1/2 complexes engage virtually the entire cytosolic pool of Sse1p, suggesting that the majority, if not all, of Sse's cellular functions lie within complexes with these Hsp70s. These interactions regulate the nucleotide cycles of the involved Hsp70s, as Sse1p is a potent nucleotide exchange factor for both Ssa1p and Ssbp1 (B.B. and F.-U. Hartl, unpublished data). An intriguing speculation is that the NEF activity of Sse1p allows for a direct substrate handover from Ssa1p or Ssb1/2p onto Sse1p for further folding assistance.

A further, even more involved, interaction exists between two ribosome-associated Hsp70s, Ssb and Ssz, which interact with each other via a third protein, a J protein known as zuotin (Figure 2). Ssb physically associates with nascent chains at the yeast ribosome and appears to be involved with early folding events and perhaps in the translation process itself. It has long been known to interact with zuotin. Enabling this pairing, remarkably, is Ssz, known to associate with zuotin in a stable manner, which is unusual for Hsp70 interactions with J proteins (Gautschi et al., 2001). Yet Ssz, by contrast with Ssb, does not associate with nascent polypeptide chains—in fact, its C-terminal SBD can be deleted without compromising its activity in vivo (Hundley et al., 2002). Huang et al. (2005) report that although Ssz can bind nucleotides, it lacks ATP hydrolysis activity almost completely. In addition, mutations in the NBD of Ssz that are predicted to interfere with ATP binding or hydrolysis do not affect the in vivo activity of Ssz. Consistent with this, in vitro, zuotin stimulates the ATPase activity of only Ssb and not Ssz. Thus, it seems that Ssz is an Hsp70 stripped of the usual functions except that of binding zuotin. Zuotin, for its part, is the first example of a J protein which has to physically associate with one Hsp70, Ssz, in order to stimulate the ATPase of another Hsp70, Ssb. This ménage a trois nicely explains the genetic data, which demonstrate that mutants lacking Ssb, Zuo1, or Ssz1 have similar phenotypes. The interactions are apparently not restricted to yeast cells, as it has been recently demonstrated that the J protein MPP11 and Hsp70L1 form a ribosome-associated complex in mammalian cells (Otto et al., 2005), with MPP11 cooperating with cytosolic Hsc70 (Hundley et al., 2005). However, how and why this unusual mechanism of activation of Ssb/Hsc70 operates remain open questions. More generally, it will be interesting to unravel the biological importance and mechanisms of Hsp70-Hsp70 interactions.

AAA+ Unfoldase Rings—Moving Parts and Action Coordinated by ATP

Hexameric chaperone rings, whose barrels are composed of AAA+ ATPase modules, are involved with ATP-mediated unfolding of proteins in such contexts as protein degradation, protein disassembly, and protein trafficking across membranes (Sauer et al., 2004). For example, in preparation for proteasomal degradation, a hexameric ring of AAA+ ATPases at the base of the 19S particle of the proteasome unfolds proteins and translocates them into the cylindrical 20S protease core. Similarly, homomeric complexes of bacterial ClpX and ClpA unfold and translocate substrates into the cylindrical serine protease ClpP. In protein disaggregation, the ClpB chaperone in bacteria and Hsp104 in the yeast cytosol act in the absence of any partner protease to pry apart protein aggregates. And in trafficking, p97 pulls on proteins during retrotranslocation from the ER as one of its several actions, whereas the N-ethylmaleimide-sensitive factor (NSF) assembly pulls apart helical-bundle SNARE complexes to enable SNARE components to participate in vesicle fusion. Recent work begins to elucidate one mechanism by which the energy of ATP hydrolysis can be translated by these machines into the exertion of mechanical force needed for unfolding.

The loops within the central channel of AAA+ unfoldase rings that bind substrate proteins may be a moving part of these machines. Structural and functional studies suggest that the loops translocate bound segments of protein axially down the channel in response to ATP hydrolysis, exerting a mechanical pulling force. This pulling action is associated with unfolding because the substrate protein is forced to enter a narrow channel that cannot otherwise be negotiated. In the case of ClpA, this channel measures $\sim$12 Å in diameter, a bore that cannot accommodate much more than an α-helical secondary structure. Repeated rounds of pulling action thus ultimately unravel a protein's structure, commencing from the point that was initially recognized (Lee et al., 2001).

Evidence for loop movement came first from X-ray work on two hexameric nucleic-acid translocases, one a packaging motor that drives a double-stranded RNA genome into the phi12 phage head (Mancini et al., 2004), and the other the SV40 T antigen, which separates viral DNA strands at the origin by translocating them down its channel and out through side holes (Gai et al., 2004). Both machines were crystallized in different nucleotide bound states, revealing striking differences in the position of channel-facing loops, with a proximal position found in ATP-like states and a distal position observed in ADP bound ones. Remarkably, when similar nucleo-

tide bound structures of the intact p97 cylinder were examined, a channel-facing loop belonging to the distal AAA+ ATPase module (D2) exhibited the same movement (DeLaBarre and Brunger, 2005; Figure 3). In parallel, substrate crosslinking and mutation studies with ClpA identified loops in its central channel at the level of both its D1 and D2 domains that mediate substrate binding; most interestingly, one substitution mutant, immediately flanking the large D2 loop, could bind substrate but could not mediate unfolding and ClpP-mediated degradation, implicating this loop in translocation and unfolding (Hinnerwisch et al., 2005).

There are interesting wrinkles to this model of the action of unfoldases. Homologous loops have also been implicated in substrate binding inside the channel of both ClpX and ClpB. In ClpB, this implies that its protein disaggregation activity is potentially associated with the unfolding that occurs during translocation down its central channel. This was supported by an experiment in which the distal surface of ClpB was engineered so that it interacted with the ClpP protease. This version of ClpB promoted the degradation of disaggregated substrates (Weibezahn et al., 2004).

The action of p97 is also interesting to consider. Here, X-ray studies show only a narrow channel opening at the level of the D1 domain in all of the states examined, which appears too small for polypeptide passage, raising the question of how p97 could act on its substrates (Figure 3). Is this channel opened, for example, by the binding of substrate or adaptor proteins to p97? Or are substrates obligated to both enter and exit the D2 opening of this cylinder, effectively grasped and dislocated by the D2 loops? Or do substrates never enter the p97 channel at all, such that the observed D2 loop movements are not utilized to perform work? An alternative working part might be the N-terminal domains, which are small α-β domains flexibly attached to the D1 domains near the top of the cylinder. These domains are able to bind adaptor proteins that deliver particular substrates. It seems possible that ATP-directed movement of these domains and the associated p97 adaptors, Npl4/Ufd1 or p47, could accomplish mechanical work on substrate proteins. This would alleviate a steric problem of fitting ubiquitinated proteins into the channel during retrotranslocation (or, alternatively, having to deubiquitinate and reubiquitinate substrates). In contrast with this putative mechanism, although ClpX and ClpA recognize some of their substrates via adaptors or via the N domains themselves, they definitely utilize their channel hardware. For example, they are able to recognize and act on an entire class of substrates (bearing ssrA C-terminal tags) in the complete absence of the N domains, the AAA+-formed channel alone being sufficient for binding and unfolding. Additionally, in the case of other substrates initially delivered via adaptors or first bound via the N domains, the channel loops are required for subsequent unfolding action (Hinnerwisch et al., 2005).

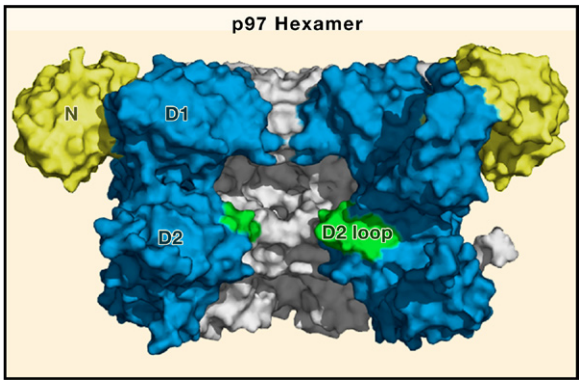

Figure 3. Crystallographic Model of an Intact p97 Hexamer
A space-filling model illustrates the architecture of the p97 ring, composed of N-terminal domains (yellow) and stacked D1 and D2 AAA+ modules (blue). The mobile channel-facing loops, referred to as the D2 loops, are shown in green. They shift from an "up" position in ATP-like states to a "down" one in ADP. Note also the narrowing of the channel at the level of the D1 domains (see text).

The Coordinated Action of ATP

The question of how ATP hydrolyzes around a hexameric ring in order to carry out the work of unfolding has begun to be addressed. A nonconcerted action appears to be involved. For example, in the case of a hydrolysis-defective (Walker B mutant) ClpX ring, only 3 to 4 subunits bound ATP at a time, some exhibiting slow dissociation of ATP and others rapid, suggesting that any given subunit could occupy at least three different states during the ATP reaction cycle (Hersch et al., 2005). When various combinations of wild-type and mutant ClpX AAA+ modules were introduced into rings by putting six coding sequences in tandem (by placing a linker outside the ring between neighboring subunits) a single ATP-proficient subunit could enable unfolding and ClpP-mediated degradation of a structured substrate, albeit at a rate only 2% that of wild-type ClpX (Martin et al., 2005). Intriguingly, addition of a second wild-type subunit at the opposite side of the ring increased the rate to 30% of wild-type. This indicates that the use of more than one subunit is significantly favored, but whether such action is concerted or sequential remains to be seen. However, given that many different arrangements were functional, a strict geometric progression seems to be excluded. A "probabilistic" model was proposed that would activate hydrolysis in a subunit based on the state of adjacent subunits or interaction of the subunit with substrate. As yet, there is no evidence that substrate itself triggers hydrolysis in the subunit to which it binds, but presumably it is the hydrolysis of ATP within subunits that have bound substrate that carries out the mechanical work. Clearly, the probabilistic model can accommodate a substrate that is asymmetrically winding down the central channel of these machines, although a nonstringent version of a binding-change sequential mechanism, like that employed by F_1-ATPase, does not seem excluded.

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REFERENCES

Anken, E., Braakmann, I., and Craig, E. (2005). Versatility of the endoplasmic reticulum protein folding factory. Crit. Rev. Biochem. Mol. Biol. *40*, 191–228.

Arrasate, M., Mitra, S., Schweitzer, E.S., Segal, M.R., and Finkbeiner, S. (2004). Inclusion body formation reduces levels of mutant huntingtin and risk of neuronal death. Nature *431*, 805–810.

Bence, N., Sampat, R., and Kopito, R.R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. Science *292*, 1552–1555.

Bennett, E.J., Bence, N.F., Jayakumar, R., and Kopito, R.R. (2005). Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol. Cell *17*, 351–365.

Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Øvervatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. *171*, 603–614.

Cormier, J.H., Pearse, B.R., and Hebert, D.N. (2005). Yos9p: a sweet-toothed bouncer of the secretory pathway. Mol. Cell *19*, 717–719.

Credle, J.J., Finer-Moore, J.S., Papa, F.R., Stroud, R.M., and Walter, P. (2005). On the mechanism of sensing unfolded protein in the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA *102*, 18773–18784.

DeLaBarre, B., and Brunger, A.T. (2005). Nucleotide dependent motion and mechanism of action of p97/VCP. J. Mol. Biol. *347*, 437–452.

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. *4*, 181–191.

Gai, D., Zhao, R., Li, D., Finkielstein, C.V., and Chen, X.S. (2004). Mechanisms of conformational change for a replicative hexameric helicase of SV40 large tumor antigen. Cell *119*, 47–60.

Gautschi, M., Lilie, H., Funfschilling, U., Mun, A., Ross, S., Lithgow, T., Rucknagel, P., and Rospert, S. (2001). RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin. Proc. Natl. Acad. Sci. USA *98*, 3762–3767.

Harrison, C.J., Hayer-Hartl, M., DiLiberto, M., Hartl, F.-U., and Kuriyan, J. (1997). Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. Science *276*, 431–435.

Helenius, A., and Aebi, M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. Annu. Rev. Biochem. *73*, 1019–1049.

Hersch, G.L., Burton, R.E., Bolon, D.N., Baker, T.A., and Sauer, R.T. (2005). Asymmetric interactions of ATP with the AAA+ $ClpX_6$ unfoldase: allosteric control of a protein machine. Cell *121*, 1017–1027.

Hinnerwisch, J., Fenton, W.A., Farr, G.W., Furtak, K., and Horwich, A.L. (2005). Loops in the central channel of ClpA chaperone mediate protein binding, unfolding, and translocation. Cell *121*, 1029–1041.

Huang, P., Gautschi, M., Walter, W., Rospert, S., and Craig, E.A. (2005). The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1. Nat. Struct. Mol. Biol. *12*, 497–504.

Hundley, H., Eisenman, H., Walter, W., Evans, T., Hotokezaka, Y., Wiedmann, M., and Craig, E. (2002). The in vivo function of the ribosome-associated Hsp70, Ssz1, does not require its putative peptide-binding domain. Proc. Natl. Acad. Sci. USA *99*, 4203–4208.

Hundley, H., Walter, W., Bairstow, S., and Craig, E.A. (2005). Human Mpp11J protein: ribosome-tethered molecular chaperones are ubiquitous. Science *308*, 1032–1034.

Iwata, A., Christianson, J.C., Bucci, M., Ellerby, L.M., Nukina, N., Forno, L.S., and Kopito, R.R. (2005). Increased susceptibility of cytoplasmic over nuclear polyglutamine aggregates to autophagic degradation. Proc. Natl. Acad. Sci. USA *102*, 13135–13140.

Jiang, J., Prasad, K., Lafer, E.M., and Sousa, R. (2005). Structural basis of interdomain communication in the Hsc70 chaperone. Mol. Cell *20*, 513–524.

Kimata, Y., Oikawa, D., Shimizu, Y., Ishiwata-Kimata, Y., and Kohno, K. (2004). A role for BiP as an adjustor for the endoplasmic reticulum stress-sensing protein Ire1. J. Cell Biol. *167*, 445–456.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., et al. (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. *169*, 425–434.

Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhisa, T., and Mizushima, N. (2004). The role of autophagy during the early neonatal starvation period. Nature *432*, 1032–1036.

Lee, C., Schwartz, M.P., Prakash, S., Iwakura, M., and Matouschek, A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol. Cell *7*, 627–637.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: Molecular mechanisms and biological functions of autophagy. Dev. Cell *6*, 463–477.

Mancini, E.J., Kainov, D.E., Grimes, J.M., Tuma, R., Bamford, D.H., and Stuart, D.I. (2004). Atomic snapshots of an RNA packaging motor reveal conformational changes linking ATP hydrolysis to RNA translocation. Cell *118*, 743–755.

Martin, A., Baker, T.A., and Sauer, R.T. (2005). Rebuilding a hexameric AAA+ motor reveals operating principles for ATP-fueled machines. Nature *437*, 1115–1120.

Molinari, M., Calanca, V., Galli, C., Lucca, P., and Paganetti, P. (2003). Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science *299*, 1397–1400.

Montgomery, D.L., Morimoto, R.I., and Gierasch, L.M. (1999). Mutations in the substrate binding domain of the Escherichia coli 70 kDa molecular chaperone DnaK which alter substrate affinity or interdomain coupling. J. Mol. Biol. *286*, 915–932.

Nishikawa, S., Brodsky, J.L., and Nakatsuka, K. (2005). Role of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). J. Biochem. (Tokyo) *137*, 551–555.

Oda, Y., Hosokawa, N., Wada, I., and Nagata, K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science *299*, 1394–1397.

Otto, H., Conz, C., Maier, P., Wolfle, T., Suzuki, C.K., Jeno, P., Rucknagel, P., Stahl, J., and Rospert, S. (2005). The chaperones MPP11 and Hsp70L1 form the mammalian ribosome-associated complex. Proc. Natl. Acad. Sci. USA *102*, 10064–10069.

Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J., and Rubinsztein, D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. *36*, 585–595.

Romisch, K. (2005). Endoplasmic reticulum-associated degradation. Annu. Rev. Cell Dev. Biol. *21*, 435–456.

Romisch, K. (2006). Cdc48p is UBX-linked to ER ubiquitin ligases. Trends Biochem. Sci. *31*, 24–25.

Sauer, R.T., Bolon, D.N., Burton, B.M., Burton, R.E., Flynn, J.M., Grant,

R.A., Hersch, G.L., Joshi, S.A., Kenniston, J.A., Levchenko, I., et al. (2004). Sculpting the proteome with AAA+ proteases and disassembly machines. Cell *119*, 9–18.

Schroder, M., and Kaufman, R.J. (2005). The mammalian unfolded protein response. Annu. Rev. Biochem. *74*, 739–789.

Shaner, L., Wegele, H., Buchner, J., and Morano, K.A. (2005). The yeast Hsp110 Sse1 functionally interacts with the Hsp70 chaperones Ssa and Ssb. J. Biol. Chem. *280*, 41262–41269.

Shomura, Y., Dragovic, Z., Chang, H.C., Tzvetkov, N., Young, J.C., Brodsky, J.L., Guerriero, V., Hartl, F.U., and Bracher, A. (2005). Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange. Mol. Cell *17*, 367–379.

Silveira, J.R., Raymond, G.J., Hughson, A.G., Race, R.E., Sim, V.L., Hayes, S.F., and Caughey, B. (2005). The most infectious prion protein particles. Nature *437*, 257–261.

Sondermann, H., Scheufler, C., Schneider, C., Hohfeld, J., Hartl, F.U., and Moarefi, I. (2001). Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors. Science *291*, 1553–1557.

Steel, G.J., Fullerton, D.M., Tyson, J.R., and Stirling, C.J. (2004). Coordinated activation of Hsp70 chaperones. Science *303*, 98–101.

Vabulas, R.M., and Hartl, F.U. (2005). Protein synthesis upon acute nutrient restriction relies n proteasome function. Science *310*, 1960–1963.

Vashist, S., and Ng, D.T. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J. Cell Biol. *165*, 41–52.

Venkatraman, P., Wetzel, R., Tanaka, M., Nukina, N., and Goldberg, A.L. (2004). Eukaryotic proteasomes cannot digest polyglutamine sequences and release them during degradation of polyglutamine-containing proteins. Mol. Cell *14*, 95–104.

Vogel, M., Bukau, B., and Mayer, M.P. (2006). Allosteric regulation of Hsp70 chaperones by a proline switch. Mol. Cell *21*, 359–367.

Weibezahn, J., Tessarz, P., Schlieker, C., Zahn, R., Maglica, Z., Lee, S., Zentgraf, H., Weber-Ban, E.U., Dougan, D.A., Tsai, F.T., et al. (2004). Thermotolerance requires refolding of aggregated proteins by substrate translocation through the central pore of ClpB. Cell *119*, 653–665.

Yam, A.Y., Albanese, V., Lin, H.T., and Frydman, J. (2005). Hsp110 cooperates with different cytosolic HSP70 systems in a pathway for de novo folding. J. Biol. Chem. *280*, 41252–41261.