Heart Failure and Protein Quality Control

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Abstract—The heart is constantly under mechanical, metabolic, and thermal stress, even at baseline physiological conditions, and cardiac stress may increase as a result of environmental or intrinsic pathological insults. Cardiomyocytes are continuously challenged to efficiently and properly fold nascent polypeptides, traffic them to their appropriate cellular locations, and keep them from denaturing in the face of normal and pathological stimuli. Because deployment of misfolded or unfolded proteins can be disastrous, cells, in general, and cardiomyocytes, in particular, have developed a multilayered protein quality control system for maintaining proper protein conformation and for reorganizing and removing misfolded or aggregated polypeptides. Here, we examine recent data pointing to the importance of protein quality control in cardiac homeostasis and disease. (Circ Res. 2006;99:1315-1328.)

Key Words: cardiac disease ■ cardiac failure ■ cardiac muscle ■ cardiomyocytes ■ cardiovascular disease ■ cardiovascular physiology

Because of alternative splicing of primary transcripts, posttranslational modifications, and the ability to assume multiple conformations that differ in activity, the proteome, in terms of its informational content, is considerably more complex than the genome and transcriptome. Thus, it is not surprising that controlling the quality of this information is essential for cell survival and function. Multiple layers of quality control for protein production and maintenance exist. After their initial synthesis, proteins targeted for the membrane and secretory pathways are modified, folded, and assembled in the endoplasmic reticulum (ER), whereas other cellular proteins may be synthesized and processed independently of the ER in the cytosol. Accordingly, there exist both ER-associated protein quality control (PQC) and ER-independent PQC. In both cases, molecular chaperones and the ubiquitin/proteasome system (UPS) play essential roles. In general, chaperones are responsible for protecting unfolded or partially folded nascent or mature proteins, with many chaperones participating in protein repair, whereas the UPS is largely responsible for removing terminally misfolded proteins permanently, thereby preventing misfolded proteins from accumulating in the cell.

Although the first lines of defense rest in the “proofreading” of the primary DNA and RNA sequences, the cell has evolved multiple layers of control at the posttranslational level as well, and nascent proteins are subject to rigorous surveillance as they are synthesized on the polysomes. Although small- and medium-sized proteins often can assume their correct tertiary and quaternary conformations spontaneously, the majority of proteins cannot and depend on the help of and interaction with other proteins to fold correctly. The complete sequence is often necessary for assuming the correct conformation, but the linear process of protein synthesis presents unfinished proteins to the cellular environ-
Small heat shock proteins, and a class of chaperones whose synthesis is induced in response to stress, have protective roles in the heart and the nomenclature, and their interaction and subsequent aggregation. The complexity of this process and the diversity of peptide sequence are reflected in the large number and types of chaperones and cochaperones, such as the chaperonin family, the nuclear chaperones that help assemble nucleosomes, the mitochondrial-specific chaperones for the respiratory chain proteins, and a class of chaperones whose synthesis is induced in response to stress, the small heat shock proteins (HSPs).

The small HSPs are a class of chaperones that have been studied extensively and play important roles in normal cellular function and disease development. The “heat-shock response,” in which an entire group of genes is activated in response to environmental stress, was first described in Drosophila. The small HSPs play important chaperone and protective roles in the heart and the nomenclature, and classification of the members in the family has recently been reviewed in detail. They are abundant in cardiomycocytes and are upregulated during cardiac stress and disease, with a single member, α-B crystallin (CryAB), making up as much as 3% of the total soluble protein mass. CryAB binds both desmin and cytoplasmic actin and possesses molecular chaperone function in vitro. All of these considerations, coupled with genetic evidence linking an R120G mutation in CryAB to human cardiac disease, prompted a series of experiments demonstrating that CryAB expression was sufficient to cause heart failure in vivo.

The Ubiquitin/Proteasome System

The integrity of a protein is constantly questioned by the PQC. Damage control is particularly important in long-lived cells, such as cardiomycocytes because they normally do not proliferate and thus are relatively sensitive to increasing concentrations of misfolded protein. In this respect, they share an important feature with the neuronal populations, which also cannot proliferate under normal conditions. Ubiquitin/proteasome system (UPS)-mediated proteolysis constitutes a second line of defense for the PQC of a cell by removing misfolded, oxidized, mutant, or otherwise damaged proteins. The UPS also degrades normal proteins that are no longer needed, providing temporal regulation of protein activity. UPS-mediated proteolysis includes 2 major steps: attachment of a polyubiquitin chain via isopeptide bonds to the target protein molecule through a process known as ubiquitination and the subsequent degradation of the ubiquitinated proteins by the 26S proteasome. Both ubiquitination and proteasome-mediated degradation are highly regulated cellular processes. Although monoubiquitination does occur, polyubiquitination is the posttranslational process that targets specific proteins for degradation by the 26S proteasome. Ubiquitination is performed by a cascade of enzymatic reactions that are catalyzed by 3 classes of enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (Ubc, E2), and ubiquitin ligase (E3). Recent data indicate that polyubiquitin chain assembly factor (E4) may also play an important role, as UFD2α, an E4 exclusively expressed in cardiac muscle during mouse embryonic development, is essential for normal heart development. There is 1 ubiquitin E1, approximately 50 E2s, and more than 400 potential E3s in the human genome, depending on how these proteins are defined. To date, 2 E4s have been identified in mammalian cells.

The high efficiency and exquisite specificity of ubiquitination relies mainly on the E3 ligases. Nearly all known E3s use 1 of 2 catalytic domains: a HECT (Homologous to E6AP Carboxyl Terminus) domain or a RING (Really Interesting New Gene 1) finger domain. The U-box domain can also execute ubiquitin ligase activity. The U-box is a distant relative of the RING finger domain because it has a RING-like conformation but lacks the canonical Zn-coordinating residues. HECT domain E3s are typified by human E6AP. There are approximately 40 HECT domain–encoding genes, more than 380 RING finger protein genes, and 9 U-box genes in the human genome. They can possess ubiquitin ligase activity by themselves or as part of multisubunit E3s. HECT E3s are monomeric enzymes that directly participate in the chemistry of substrate ubiquitination reactions by forming a ubiquitin–thioester intermediate and transferring a ubiquitin monomer to the target protein molecule during each round of catalysis in the ubiquitin–isopeptide bond formation. In contrast, the RING finger domain proteins usually form a multisubunit complex with partner proteins and bring activated E2s and substrates into close proximity, facilitating ubiquitin–isopeptide bond formation.

The SCF (Skp1–Cul1–F-box protein) complexes are the prototype of a superfamily of cullin (Cul)-based RING finger–type E3s that represent the largest family of ubiquitin ligases and mediate the ubiquitination of a variety of regulatory and signaling proteins in diverse cellular pathways. The SCF consists of 3 invariant components, Skp1, Cul1, and Rbx1 (a RING finger protein, also known as Roc1 or Hrt1) and an interchangeable F-box protein subunit. Cul1–Rbx1 forms the catalytic core of the complex and is responsible for recruiting E2, whereas Skp1 serves as an adaptor that is able to bind different F-box proteins. The F-box proteins, carrying a Skp1-interacting F-box motif and a protein–protein interaction domain, can dock different protein substrates that are often phosphorylated to the SCF complex for ubiquitination. In addition to Cul1, the cullin protein family has at least 6 additional closely related paralogs in humans (Cul2, -3, -4A, -4B, -5, and -7). Cul-1 is required for cell cycle exit in Caenorhabditis elegans and is part of a novel gene family. All of these cullins can bind Rbx1 and form ubiquitin ligases. Thus, a large number of ubiquitin E3s are cullin dependent, and cullin appears to be a focal point for regulation of ubiquitin E3 assembly by neddylation, a posttranslational modification similar to ubiquitination (see below).

The carboxyl terminus of the Hsc70-interacting protein (CHIP), originally identified as a cochaperone of Hsc70, is also a ubiquitin E3 ligase. It has both a tetraubiquitopeptide repeat motif and a U-box domain. The tetraubiquitopeptide repeat
Figure 1. Proteasome complexes. Shown are the various particles and their subunit compositions.

Proteasomes

The 26S proteasome is the cellular machinery responsible for degrading polyubiquitinated proteins. The 26S is composed of a 20S proteolytic core particle (the 20S proteasome) and a proteasome activation particle (the 19S proteasome), which is located at 1 or both ends of the 20S proteasome. The 20S is a hollow cylindrical structure formed by a stack of 4 heptameric rings: 2 central antiparallel identical α rings and 2 outer α rings (Figure 1). Each α or β ring consists of 7 unique protein molecules (α1 to α7, β1 to β7). The unfolded protein is degraded in the cavity of the 20S by the chymotrypsin-like, trypsin-like, and caspase-like (also known as peptidylglutamyl-peptide hydrolase) activities, which reside in the β5, β2, and β1 subunits, respectively. The outer α ring forms the gate, which regulates substrate entry into the proteolytic chamber and also controls the exit of the proteolytic peptide products.12

The 19S (also known as PA700) binds to the 20S through α-ring interactions. The 19S from Saccharomyces cerevisiae consists of at least 17 subunits: regulatory particle non-ATPase (Rpn) 1 to 12 and regulatory particle tripleA-ATPase (Rpt) 1 to 6. The 6 tripleA-ATPase subunits, along with Rpn1 and Rpn2, form the base of the 19S, whereas Rpn3, Rpn5 to Rpn9, Rpn11, and Rpn12 form its lid, with Rpn10 linking the lid and base. The base directly interacts with the α ring of the 20S. In murine hearts, an alternatively spliced isoform of Rpn10 has been described.42 The 19S recognizes ubiquitinated protein molecules, deubiquitates and unfolds them, and channels the unfolded protein to the 20S where peptide cleavage takes place. The proteasome also plays an important role in antigen processing and antigen presentation through the class I major histocompatibility complex. Certain conditions, such as viral infection or induction with interferon γ or tumor necrosis factor, remodel the 20S, resulting in the constitutive proteasome subunits β5, β2, and β1 being replaced with the inducible β5i, β2i, and β1i proteasome subunits, respectively. Remodeling can alter proteasome peptidase activity and proteomic analyses show that purified murine cardiac 26S proteosomes contain both the 3 constitutive and 3 inducible proteasome subunits.42

The 20S can also associate with the 11S proteasome.43 The 11S is also known as proteasome activator 28 (PA28) or REG because its subunits, PA28α, PA28β, and PA28γ, with molecular masses of approximately 28 kDa, have the capacity to enhance the peptidase activities of the 20S.45 Of the 3 PA28s, PA28α and PA28β assemble into heteroheptamers (α3/β4 or α4/β3) and PA28γ forms homoheptamers. Both types of 11S proteasomes have a molecular mass of ≈200 kDa.43 All 3 PA28s are expressed in cardiomyocytes. PA28α and PA28β are found in the cytoplasm and the nucleus, but PA28γ is located predominantly in the nucleus.44 The 20S can be capped by 19S or 11S proteasomes at both ends (19S-20S-19S or 11S-20S-11S) or by the 19S at one end and the 11S at the other (19S-20S-11S) (Figure 1). Because the 11S does not contain ATPase activity and it can enhance the peptidase activities of the 20S, it has been hypothesized that it enhances 20S proteasome peptide cleavage but does not facilitate substrate entry. However, a recent study showed that PA28γ directs degradation of the steroid receptor coactivator SRC-3 by the 20S in an ATP- and ubiquitin-independent manner, indicating that entry may be affected.44,45 In contrast to data obtained from red blood cells and liver cell preparations, Gomes et al recently reported that 11S proteasomes were not detected in murine cardiac 20S proteasome preparations and were only occasionally detected in the 26S proteasome preparations, although PA28α is fairly abundant in the heart.42 It is possible that the purification procedures selectively leave out 11S-associated 20S proteasomes. Using gel filtration, a significant amount of 20S proteasomes coeluted with the 11S proteasome in native murine myocardial proteins. Consistent with the hypothesis that this pathway plays an important role in cardiomyocyte function, a substan-
tial subset of 20S proteasomes appears to associate with the sarcomere in striated muscle and display a characteristic striated pattern when immunolabeled in striated muscle.\textsuperscript{42,46} The physiological significance of this association has not been established, but it is conceivable that the proteasome may be involved in sarcomere genesis and maintenance. In the future it will be important to define the (patho)physiological role of 11S proteasome in the heart.

**Regulation of Proteasomal Proteolytic Activity**

The potential of therapeutic manipulation of protein degradation in a specific organ or tissue type has been recognized\textsuperscript{47,48} but is limited by an incomplete understanding of how proteasomal specificity and activity are regulated. Polyubiquitination is almost always required for a specific protein to be degraded by the 26S proteasome.\textsuperscript{12} Thus, the decision for a protein to be degraded by the UPS is made by exposure or maturation of its ubiquitination signal (degron) to its specific E3 ligase. Directly enhancing proteasomal activity would unlikely increase degradation of normal proteins because posttranslational modification within or nearby the degron of a normal protein is usually required for the binding of a specific E3 complex and ensuing degradation of the normal protein.\textsuperscript{20} Taking the opposite approach, inhibition of the 20S proteasome would affect degradation of the majority of cellular proteins, whereas targeting a specific E3 ligase would likely affect a family of proteins. The most precise approach would be to manipulate the ubiquitination signal of a normal protein.\textsuperscript{20} Taking the opposite approach, inhibition of the 20S proteasome would affect degradation of the majority of cellular proteins, whereas targeting a specific E3 ligase would likely affect a family of proteins. The most precise approach would be to manipulate the ubiquitination signal of the target protein. An example of the general approach is the cullin-based ubiquitin E3 inhibitor nutlin, which specifically binds to the target protein. An example of the general approach is the cullin-based ubiquitin E3 inhibitor nutlin, which specifically binds to the target protein.

As described earlier, the binding of 19S (PA700) or 11S (PA28) proteasomes to the 20S activates the proteasome. Although 11S binding does not require ATP, the association of the 19S and 20S particles to form the 26S proteasome is ATP dependent. Unfolding the substrate protein and channeling it into the 20S proteasome by the base of 19S proteasomes also requires ATP hydrolysis.\textsuperscript{12} Thus, an overall negative energy balance, which can occur in some cardiac disease, might inhibit UPS proteolytic function directly.

The 20S proteasome appears to be able to selectively recognize and degrade oxidized proteins in the heart in a ubiquitin-independent manner.\textsuperscript{15,49} Protein oxidation often leads to denaturation and/or partial unfolding, exposing hydrophobic surfaces or domains that are normally buried in the native polypeptide. Proteasomes have a relatively high affinity for hydrophobic amino acids,\textsuperscript{50} and, thus, partially denatured proteins that have undergone mild oxidation may well be preferentially bound by the 20S proteasome and targeted in a ubiquitin-independent manner for degradation.\textsuperscript{51} It should be emphasized, however, that this activity is dependent on only mild oxidation, and if significant oxidative injury takes place, such as what occurs during a major cardiac ischemic event, proteasomal proteins themselves are significantly damaged and proteasomal activity decreased.\textsuperscript{52,53} Proteasomal dysfunction during ischemia has been extensively discussed.\textsuperscript{13} The exact mechanism of this inhibition remains to be determined as the ischemic cell will also be ATP deficient, and, thus, the ATP-dependent ubiquitination pathways will also be affected.

Posttranslational modifications, including phosphorylation, N-terminal acetylation, and N-terminal myristoylation, are observed in proteasome subunits.\textsuperscript{15,54} but their functional significance is largely undefined. The α3 and α7 subunits in 20S proteasomes isolated from Rat-1 fibroblasts and human embryonic lung cells (L-132) are phosphorylated. Following dephosphorylation by acidic phosphatase, immunoprecipitated proteasomes displayed a significantly lower activity compared with the phosphorylated proteasomes.\textsuperscript{55} The α7 subunit is phosphorylated by proteasome-associated casein kinase II (CK2) at Ser243 and Ser250.\textsuperscript{56} Phosphorylation of α7 may play an important role in stabilizing the 26S proteasome.\textsuperscript{57} Several subunits of the 19S particle in animal cells are also phosphorylated, and this may be important in mediating 26S proteasome assembly.\textsuperscript{58,59} Using a proteomic approach, Ping and colleagues detected N-terminal acetylation of 19S subunits (Rpn1, Rpn5, Rpn6, Rpt3, and Rpt6) and 20S subunits (α2, α5, α7, β3, and β4), N-terminal myristoylation of a 19S subunit (Rpt2), and phosphorylation of 20S subunits (eg, α7).\textsuperscript{42} They also identified 2 previously unrecognized functional partners in the endogenous intact cardiac 20S proteasomes: protein phosphatase 2A (PP2A) and protein kinase A (PKA).\textsuperscript{54} Multiple individual subunits of 20S (eg, α1 and β2) appear to be the targets of PP2A and PKA; inhibition of PP2A or the addition of PKA significantly modified both the serine- and threonine-phosphorylation profile of proteasomes, and phosphorylation of the 20S complex enhances the peptidase activity of the individual subunits in a substrate-specific fashion. Taken together, these studies show that the peptidase activities of cardiac 20S proteasomes are modulated by associating partners and phosphorylation may be a key mechanism for regulation.\textsuperscript{15}

**The COP9 Signalosome**

The COP9 signalosome (CSN) can regulate UPS function. In mammalian cells, the CSN is a multimeric protein complex consisting of 8 unique protein subunits, referred to as CSN1 through CSN8.\textsuperscript{25} The composition of the CSN complex and the domain structure of its subunits resemble the 8 subunit–containing lid subcomplex of the 19S proteasome.\textsuperscript{27} Its essential role in mammalian development is confirmed by embryonic lethality in csn2, csn3, and csn5 nulls.\textsuperscript{60} The biochemical activities and cellular functions of CSN in mammalian systems remain obscure, but studies in lower organisms point to its importance in controlling overall proteasomal activity. A major target of the CSN is the cullin-based ubiquitin E3 ligase complex. Cullin family members are hydrophilic proteins that were initially characterized as being involved in the control of yeast cell division control and can be covalently modified by a ubiquitin-like protein, Nedd8/Rub1, via a process known as neddylation, which is similar to ubiquitination. The CSN is responsible for the cleavage of the Nedd8 moiety (ie, deneddylation) from cullins. CSN-associated deubiquitination activities have also been described.\textsuperscript{61,62} These activities can be subdivided into the ability to deconjugate ubiquitin from monoubiquitinated substrates as well as depolarization of polyubiquitin.\textsuperscript{62} Col-
lectively, the CSN has both de neddylation and deubiquitination activity, either by possessing the intrinsic catalytic activity or by selectively recruiting different enzymes under different circumstances.

The lid of the 19S proteasome consists of 8 subunits that are paralogs of the 8 CSN subunits. Both CSN and the lid of 19S proteasomes contain 6 subunits with a PCI (Proteasome, COP9, eIF3) domain and 2 subunits with a MPN (MOV34, PAD N-terminal) domain. CSN and the lid of the 19S also have a similar architecture. Recent studies indicate that CSN directly interacts with the 26S proteasome and may compete with the lid of the 19S proteasome, thereby in uencing proteasomal activity. Both puri ed CSN and the lid of 19S proteasomes can bind polyubiquitin chains in vitro. Although it is likely that CSN directly regulates the proteolytic function of the proteasome, this has not been demonstrated directly and it will be important to formally test CSN ability to act cooperatively with the ubiquitination machinery to achieve ubiquitin-dependent degradation of speci c regulators important for cardiac function.

Assessment of UPS Proteolytic Function

Synthetic uorogenic peptide substrates are widely used to determine the chymotrypsin-like, trypsin-like, and caspase-like activities of the 20S proteasome in either tissue and cell lysates or in puri ed proteasomes. However, there are a number of technical concerns that limit this assay. Because these substrates can also be cleaved by nonproteasomal peptidases, only the portion of peptidase activities inhibited by a speci c proteasome inhibitor can be attributed to the proteasome. An additional concern is that the synthetic peptide substrates are usually very small and can diffuse into the 20S proteolytic chamber, even in the absence of proteasome activators such as the 19S and the 11S proteasomes. Thus, although uorogenic peptidase assays permit rapid assessment of the catalytic activity of the 20S proteasome, they may not accurately re ect UPS proteolytic function and neither the ubiquitination step nor the highly regulated entry of substrates into the 20S proteasome is e ectively evaluated by these assays.

To better assess UPS proteolytic function in cells, tissues, and the whole animal, a series of uorescence protein reporters were developed. Biological reporters such as green uorescence protein (GFP) or luciferase were modi ed such that they were targeted for ubiquitination and degradation by the UPS. These reporters use di erent ubiquitination signals, such as a noncleavable ubiquitin (Ub) fusion construct (e.g., Ub-GFP, a aavable Ub fusion peptide that permits the creation of an N-end rule substrate (e.g., Ub-Arg-GFP), and the CL1 degron (GFP*) (Figure 2).60,61 Reporters carrying di erent ubiquitination signal sequences conceivably could detect di erent ubiquitin conjugation pathways traversed en route to the proteasome. An example of this general strategy is illustrated by the degron CL1, a peptide sequence consisting of 16 amino acids (ACKNWFSSLH-FVIHL). Structural predictions indicate that the CL1 peptide sequence can form an amphipathic helix with surface exposure of a patch of hydrophobic amino acids, which may be a structural feature that is shared by misfolded proteins and signals for ubiquitination. The fusion of CL1 to the carboxyl terminus of GFP creates GFP* (or GFPu). GFP* is a speci c UPS substrate in cultured human embryonic kidney (HEK) cells and neonatal rat ventricular myocytes.65 In HEK cells, GFP* has a half-life of ~30 minutes, which is much shorter than unmodi ed GFP. Cell lines stably expressing GFP* and recombinant adenoviruses capable of delivering the GFP* transgene have been successfully used.67,68

Transgenic (Tg) mouse lines that ubiquitously express a similarly engineered, but a slightly different GFP referred to as GFPdgn, have been made.73 The GFPdgn mice were used for monitoring dynamic changes in UPS proteolytic function in vivo. Systemic inhibition of the proteasome by pharmacological agents such as MG-262 and lactacystin resulted in signi cant accumulation of GFPdgn in all organs examined, including the heart.73 Interestingly, a similar approach directed at inhibiting the proteasome did not lead to accumulation of a di erent UPS reporter (UbG76V-GFP) in UbG76V-GFP Tg mouse hearts, skeletal muscle, and brain, suggesting that GFPdgn mice are better suited for monitoring UPS proteolytic function in these organs.66 GFP* and GFPdgn are distributed in both the cytoplasm and the nucleus. Recently, nuclear and cytoplasmic variants of GFP* reporters were created by inserting either a nuclear localization sequence (NLS) or a nuclear export sequence (NES) at the N-terminus of a tandem GFP construct. The nuclear and cytoplasmic GFP* reporters (NLS-GFP* and NES-GFP*) can be used to

![Figure 2](http://circres.ahajournals.org/)

Schematic diagrams of the GFP reporters for UPS proteolytic function. A, C-terminal fusion of the degron CL1 sequence renders an enhanced GFP (EGFP) as a speci c substrate for the UPS. The EGFP signal is inversely correlated to UPS function. GFPdgn and GFP* distribute to the cytoplasm and the nucleus, serving as reporters for both compartments. NES-GFP*, a reporter for cytoplasmic UPS, was engineered by fusion of 2 EGFPs in tandem, insertion of a nuclear exclusion sequence (NES) at the N-terminal of each EGFP, and C-terminal fusion of the degron CL1. NLS-GFP*, a reporter for nuclear UPS, was made similarly, but a nuclear localization sequence (NLS) rather than NES was used. B, N-terminal fusion of a wild-type ubiquitin (Ub) to an unmodi ed GFP does not change GFP protein stability but creates an N-end rule degradation signal for the UPS if the ﬁrst amino acid of GFP is mutated from Met to Arg, rendering Ub-R-GFP a reporter for the N-end rule degradation by the UPS. N-terminal fusion of a modi ed Ub with a substitution of its ﬁnal Gly with Val (UbG76V) creates UbG76V-GFP, which is degraded by the UPS. Glycine (K) residues at positions 3 and 17 of GFP are potential ubiquitination sites (italics).
investigate localized UPS changes that may affect nuclear or cytoplasmic UPS function differently.70,74 Both reporter mice have been used to investigate UPS proteolytic function in several mouse models of human disease, and more extensive use of these fluorescence protein reporters will facilitate the study of UPS proteolytic function in cardiac physiology and pathology.73,75–77

**ER-Associated Quality Control**

In eukaryotic cells, the ER is the factory for processing and assembly of the secretory pathway proteins, which include proteins destined to the extracellular space, plasma membrane, and the exo/endocytic compartments. Nascent unfolded polypeptides are translocated into the lumen of ER, where a specialized set of enzymes and chaperones controls their posttranslational modification, helps the newly imported polypeptides to assume their native structures, and mediates their assembly into multimeric complexes. Although estimates have been made that this pathway processes ≈25% of the protein complement, it should be emphasized that, in the cardiomyocyte, the myofibrillar protein complement embodies the majority of the protein complement of the cell and is composed of proteins not processed in the ER. Thus, ER-independent PQC will play a particularly critical role in these cells. Unfortunately, ER-independent PQC has not been studied intensively, and little is known about the specific pathways underlying ER-independent quality control. Because the UPS and many molecular chaperones exist in the cytosol,30 it is reasonable to assume that ER-independent quality control is partially mediated by the collaboration between molecular chaperones and UPS-mediated proteolysis.

On exposure to aqueous solvent, hydrophobic segments in unfolded or partially folded proteins tend to aggregate. BiP/Kar2p, a member of the Hsp70 family, and other ER-resident chaperones bind to the hydrophobic patches, prevent aggregation, and preserve the folding competence of nascent peptide chains.1 Protein folding and maturation in the ER is essential for their subsequent transport through the secretory pathway. To prevent misfolded or unassembled proteins from being secreted, the ER contains a quality control system that recognizes and disposes of these proteins. ER-associated degradation is an important level of quality control in most cells78 and depends on specific retrotranslocation of aberrant proteins across the ER membrane to the cytosol, where they are degraded by the UPS. This subject has been the topic of a number of recent reviews, and the reader is referred to those for discussion and schematic depictions of the processes involved.1,78

Alterations in homeostasis by various cellular stressors can lead to a condition known as ER stress, which causes accumulation of misfolded or unfolded proteins in the ER. In response, the cell initiates a series of fundamental changes in gene expression, protein synthesis, and protein degradation, termed the unfolded protein response (UPR).79 Components of the UPR include transcriptional induction of UPR target genes (eg, ER-resident chaperones), inhibiting translational initiation and degrading existing mRNAs, which attenuates protein synthesis. Activation of the ER-associated degrada-
Consequences of Protein Misfolding and Compromised PQC in the Heart

For structures like sarcomeres, where the stoichiometry among their constituents is highly maintained, the synthesis and incorporation of protein must be accurately coupled with the degradation of the existing protein. Myofibrillar protein degradation is thought to occur via the UPS. Insufficiency in the molecular chaperones needed to mediate folding of the large sarcomeric components or UPS malfunction could affect either nascent protein assembly or efficient removal of abnormal proteins. These processes could then result in abnormal protein accumulation and aberrant protein aggregation that could further impair the UPS and PQC. If the UPS is compromised or overloaded, what are the consequences for a cell that is terminally differentiated and cannot divide? In neurons, the consequences are well defined and protein misfolding is known to underlie a number of the neurodegenerative diseases. Very often, the common pathological characteristic is the presence of distinct bodies in the affected regions. These bodies can be extracellular or intracellular and contain aggregates of misfolded proteins that are often ubiquitinated but have failed to be targeted to the proteasome for degradation. The intracellular aggregates, which are generically referred to as inclusion bodies, are intractable to refolding and dissolution. These insoluble and kinetically stable entities contain common components such as desmin, ubiquitin, and tubulin. In some cell types such as neurons, the misfolded proteins are attached to dynein motors, transported in a retrograde manner along the microtubules to a perinuclear location and coalesce into well-defined, electron dense bodies known as aggresomes.

In the heart, the direct consequence of inadequate PQC is accumulation of misfolded proteins and aberrant protein aggregation, which also are characteristic for the desmin-related cardiomyopathies. Desmin-related cardiomyopathy is an important component of desmin-related myopathy (DRM), which is a heterogeneous group of human myopathies characterized by the presence of abnormal intrasarcoplasmic protein aggregates that are desmin positive. Although abnormal desmin-reactive material in affected muscle cells is a hallmark of DRM, a number of other proteins also accumulate, including dystrophin, ubiquitin, nestin, vimentin, CryAB, and lamin-B. These accumulations are associated with a variable degree of myofibrillar degeneration; therefore, DRM is also referred to as myofibrillar myopathy. Clinically, DRM can present as a generalized myopathy, and restrictive, hypertrophic, and dilated cardiomyopathies have all been reported. Mutations in the desmin, selenoprotein N, myotilin, and CryAB genes have now been characterized as causative for DRM. The desmin and CryAB mutations have been modeled in mice using cardiomyocyte-specific transgenesis, and their expression led to cardiac disease. A defining characteristic of the human DRMs is the presence of intracellular, electron-dense granulofilamentous bodies detectable via transmission electron microscopy, and both Tg models showed aberrant intrasarcoplasmic and electron-dense aggregates that were desmin positive and characteristic of human DRM morphology.

These bodies were reminiscent of aggresomes, as they contained desmin, CryAB, and other aggresomal proteins. Subsequently, the electron-dense bodies present in the cardiomyocytes expressing CryAB were confirmed as being aggresomes, based on protein composition, time course of formation, and the dependence of perinuclear localization on microtubule-mediated transport. However, whether these bodies were inherently cytotoxic, benign, or even protective was unclear. In some systems, aggresomes are clearly associated with pathogenesis, but there are equally compelling data that argue for cytotoxic effects. Concentrations of aggregated, misfolded proteins could easily interfere with either cell metabolism or the inherent function of a cardiomyocyte, which depends on repeated cycles of unimpeded contraction and relaxation. As the aggregates can fill a significant volume of the cytosol and deform the cell and/or nucleus, it is not difficult to imagine these bodies could be inherently toxic. On the other hand, aggresomal formation could represent an attempt by the cell to sequester potentially cytotoxic, misfolded proteins from the general cytoplasm. Consistent with the latter hypothesis, preventing aggresome formation actually resulted in increased cytotoxicity in cardiomyocytes expressing CryAB.

Aggresomes are commonly found in neurodegenerative disorders that are caused by aberrant protein conformation and misfolding. Diseases that fall into this category include Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, Alexander’s disease, and the prion-based disorders. Despite some commonality of pathology, the etiologies are unique and location of the aggregates can differ. In Huntington’s disease, expansion of polyglutamine tracts results in large accumulations of intracellular aggregates containing the peptides, whereas in Alzheimer’s disease, Aβ peptide plaques and misfolded tau neurofibrillar tangles are predominantly extracellular in restricted neuronal populations. Despite the unique primary misfolded proteins responsible for these discrete neurodegenerative diseases, the pathologies are linked by the accumulation of abnormal aggregates containing a β-sheet structure, which form because of either the intrinsic mutation(s) of a protein(s) or alterations in its processing. The widespread distribution of aggresomes in diverse neurodegenerative diseases, and the appearance of aggresomes in a cardiomyocyte-based disease, prompted an examination of the possible parallels between the 2 cell types and the pathological insults to which they are subjected. Although the inability of neurons and cardiomyocytes to divide has been challenged in recent years, it is generally accepted that, for both cell types, the majority of cells are terminally differentiated and incapable of division. Thus, both might be particularly sensitive to a chronic primary pathogenic stimulus, leading to protein denaturation or misfolding if they are unable to clear the aggregates, as even a slow accumulation of misfolded proteins would eventually result in high cytoplasmic levels of potentially cytotoxic entities.
Aggresomes are typically thought of as part of the cellular response to either excess protein accumulation or as a way of sequestering misfolded proteins from the general cytoplasmic milieu if the proteasomal pathways are compromised or overloaded. UPS malfunction has long been hypothesized as an important pathogenic mechanism for the protein conformational diseases. Support for this hypothesis was buttressed by the discovery that loss-of-function mutations in genes encoding UPS components can cause neurodegenerative diseases in humans and rodents. Using a HEK cell line stably expressing the UPS reporter, GFPu, Kopito and colleagues first demonstrated that aberrant protein aggregation caused by polyglutamine-expanded huntingtin (pQ-htt), or a mutant cystic fibrosis membrane conductor protein, can impair the UPS. Almost simultaneously, Jana et al showed that cultured neuro2a expressing pQ-htt displayed aberrant protein aggregation, depressed proteasomal activity, mitochondrial dysfunction, and activation of caspases 9 and 3. Expression of a truncated myosin-binding protein C mutant linked to human familial hypertrophic cardiomyopathy also resulted in the formation of abnormal protein aggregates and impaired UPS function in cultured rat neonatal cardiomyocytes. Taken together, these reports indicate that aberrant protein aggregation caused by expression of a mutant protein can impair the UPS in cultured cells, including cardiomyocytes.

**PQC and the Formation of Soluble Preeamyloid Oligomers in Cardiomyocytes**

In addition to aggresome accumulation, the neurodegenerative disorders are characterized by peptide accumulations that contain amyloid, defined as a substance with distinct ultrastructural (10-nm fibrils with β-pleated sheets) and tinctorial (apple green birefringence and Congo red-positive staining) properties. Amyloid formation is a common theme in many neurodegenerative and protein conformational disorders, and their deposition has formed the framework for a unifying theory across the diverse disease types. Amyloidoses are associated with the formation of extracellular plaques or tangles, or intracellular inclusion bodies with amyloid-like characteristics. The neurodegenerative amyloidoses, in which the deposits are localized to diverse or specific populations of neurons, have been studied most intensively, but other nonneuropathic localized amyloidoses exist. Systemic amyloidoses are well characterized in many tissues, including the heart, and are generally thought of as a heterogeneous syndrome characterized by the formation and accumulation of extracellular proteinaceous fibrils. However, aggregate and plaque accumulation can be site specific, depending on the particular protein and for some of the neurodegenerative diseases such as Parkinson’s and Huntington’s diseases, amyloid accumulation is intracellular. In the heart, systemic nonhereditary amyloidosis is not uncommon. The syndrome is known as AL amyloidosis, in which extracellular amyloid fibrils composed of monoclonal immunoglobulin light chains accumulate. Secondary amyloidoses, termed AA amyloidosis, normally develop as a complication of chronic inflammatory disease such as occurs in patients with rheumatoid arthritis, although, for this syndrome, cardiac involvement is less common. Finally, there are a number of inherited forms of systemic amyloidoses, a common form being associated with multiple mutations in the serum protein transthyretin. The systemic amyloidoses have diverse effects on cardiac function and can result in dilated or restrictive cardiomyopathy or diastolic dysfunction.

These cardiac and neurodegenerative diseases are linked by the formation of amyloid-positive deposits, and the amyloid hypothesis, which states that amyloid accumulation is cytotoxic, has been widely accepted. However, recent data have cast doubt on the hypothesis, as there is a poor correlation between the concentration of amyloid plaques and the degree of dementia in Alzheimer’s patients. Although the inherent toxicity of the amyloid-containing aggregates has been assumed in a wide range of human conditions such as light chain amyloidosis, the spongiform encephalopathies, Alzheimer’s disease, Parkinson’s disease, and others, it is now generally thought that amyloid plaques are a consequence of a long pathogenic process and represent “tombsstones,” rather than being directly cytotoxic. Consistent with the pathogenicity of these early events in aggregate formation, significant UPS impairment occurs before the coalescence of aggregated proteins into inclusion bodies.

Thus, in the search for the primary cytotoxic entity, the focus has shifted away from extracellular accumulation of the amyloid-positive plaques to the soluble amyloidogenic peptides and the intracellular events that precede visible plaque accretion. This shift in focus is explicitly rooted in the observation that disease can precede the appearance of the classically insoluble amyloid plaques and tangles. Although many different proteins can participate in, or initiate the formation of, amyloid (see below), there are important commonalities in the amyloidogenic event, which starts with the production of a native soluble protein or peptide fragment that is inherently prone to misfolding, yielding the precursor for fibril formation. The misfolded but soluble protein can then self-associate to form soluble preamyloid oligomers (PAOs), protofibrils, and other intermediates in the amyloid fibril pathway. For example, cleavage of amyloid β-protein precursor via the action of the β and γ secretases produces Aβ protein, consisting of 40- to 42-aa residues. This peptide fragment is highly amyloidogenic and can assume an ordered, β sheet–containing conformation. These structures then contribute to the formation of small soluble oligomers that can go on to develop into protofibrils, which, in turn, self-associate into the mature amyloid fibril found in the characteristic plaques and tangles (Figure 3).

Data that Aβ protein and other amyloidogenic proteins exert their cellular toxicity as soluble PAOs and not as insoluble aggregates or fibrils have been gathered. In situ experiments, in which small soluble prefibrillar Aβ was added to mouse brain slice cultures, confirmed the neurotoxicity of the soluble protein. Amyloid oligomers were found in the cerebrospinal fluid of Alzheimer’s patients, and the soluble Aβ oligomer concentration in the human brain more accurately predicted disease severity than plaque accumulation. In Huntington’s disease, the cellular toxicity of soluble amyloid induced by the expanded glutamine repeats present in the mutated hun-
Abnormal Protein Metabolism in the Diseased Heart

Regardless of the primary cause(s), CHF is often preceded and accompanied by increased cardiomyocyte protein synthesis and hypertrophy. Invariably, this leads to increased production of abnormal proteins, which are cotranslationally degraded by the UPS. Ischemic heart disease is the most common cause of CHF. Ischemia/reperfusion injury, hypoxia, and oxidative stress all can stress the heart, affect the folding and assembly of nascent and mature polypeptides, and increase compromised protein levels. Thus, the UPR is activated in cardiac myocytes in response to hypoxia and global ischemia/reperfusion, whereas sustained ER stress inhibits UPS-mediated proteolysis of reporter proteins. Consistent with the physiological significance of these data, Martindale et al recently reported that cardiomyocyte-restricted overexpression of a tamoxifen-regulated form of ATF6 could induce ER stress–related gene expression and protect against ischemia/reperfusion injury in ex vivo mouse heart preparations. Adding to the pathological load, removal of the abnormal proteins by the proteasome can also be decreased in heart failure. The removal of abnormal proteins relies on the collaboration between chaperones and the UPS, and multiple lines of evidence (see below) suggest that UPS proteolytic function is compromised in heart failure.

Figure 3. Consequences of protein misfolding. Shown is a schematic diagram of the temporal consequences of protein misfolding. Nascent proteins interact with chaperones or small HSPs and fold correctly. However, because of genetic mutation or environmental stimuli such as mechanical stress, hypoxia or ischemia, misfolded proteins, or peptide fragments accumulate. These can aggregate into kinetically stable, insoluble entities or be recognized by the PQC, ubiquitinated, and degraded by the proteasome. Isolated regions in some of these unfolded or cleaved proteins are able to assume a β-pleated sheet structure and interact with each other to form a series of intermediate but stable structures, resulting in soluble preamyloid oligomers, which are cytotoxic. These entities can, under certain circumstances, go on to form protofibrils and may coalesce, resulting in the classic amyloid fibrils, plaques, and tangles. The arrangements of the β-strands perpendicular to the fiber axis and the β-sheets parallel to the axis in the mature amyloid fibrils are represented by the red arrows. The reversibility of many of the processes leading to mature amyloid fibril deposition is emphasized by the bidirectional arrows.
To dissect molecular mechanisms underlying cardiovascular disease induced by CryAB<sup>R120G</sup>, Chen et al determined temporal changes in UPS proteolytic function in CryAB<sup>R120G</sup> Tg mouse hearts. Ubiquitinated proteins in both the soluble fraction and total protein extracts from the heart progressively increased in CryAB<sup>R120G</sup> Tg mice, but not wild-type (WT) CryAB Tg mice, at 1 and 3 months of age, whereas free ubiquitin remained unchanged, compared with normal littermates. Ubiquitinated proteins are normally degraded efficiently by 26S proteasomes. An increase in ubiquitinated proteins indicates either proteasomal malfunction or enhanced ubiquitination. Cytosolic protein levels of both total and phosphorylated β-catenin, an endogenous substrate of the UPS, were increased in CryAB<sup>R120G</sup> hearts. To demonstrate UPS impairment, GFPdgn reporter mice were crossed with CryAB<sup>R120G</sup> or WT-CryAB Tg mice. As observed in hearts treated with specific proteasome inhibitors, GFPdgn protein was dramatically increased in CryAB<sup>R120G</sup>/GFPdgn double Tg hearts compared with GFPdgn single Tg littermates. The increase in GFPdgn protein in CryAB<sup>R120G</sup>/GFPdgn double Tg hearts was greater at 3 months than at 1 month. The increase was attributable to decreased degradation rather than increased synthesis of GFPdgn, as the steady-state GFPdgn transcript levels were not increased. Furthermore, the ability of crude protein extracts from CryAB<sup>R120G</sup> Tg hearts to degrade immunoprecipitated GFPdgn protein in vitro was significantly compromised. These data indicate that UPS proteolytic function in the heart is significantly impaired by expression of CryAB<sup>R120G</sup> protein, which misfolds and aggregates. The function of 20S proteasomes is unlikely the primary cause of observed proteasomal malfunction as their peptidase activities were actually increased, not reduced, as the hearts entered congestive failure. Further analyses suggested that the primary defect can be attributed to the compromised entry of ubiquitinated proteins into the 20S proteasome and that aberrant protein aggregation is necessary for CryAB<sup>R120G</sup> to impair UPS proteolytic function. Similar findings were obtained with another mouse model of desmin-related cardiomyopathy produced by cardiac expression of a human DRM-linked desmin mutation.

The proteasome is also compromised in animal models of pressure-overload cardiomyopathy and myocardial ischemia/reperfusion, 2 common causes of CHF. In mice with thoracic aortic constriction--induced pressure overload, Tsukamoto et al measured significant decreases in proteasomal chymotrypsin-like, trypsin-like, and caspase-like peptidase activities 2 weeks after surgery but before any discernable cardiac dysfunction. These decreases became more substantial at 4 weeks when cardiac failure became evident. Consistent with proteasome functional depression, ubiquitinated proteins significantly and progressively increased between 2 and 4 weeks after thoracic aortic constriction. Using a rat model, Bulteau et al demonstrated that myocardial ischemia/reperfusion resulted in decreased cytosolic proteasomal peptidase activity and oxidative modification of the components of the 20S proteasome. This study, along with a previous report by Okada et al, confirmed that 20S proteasomal subunits are modified under these pathological conditions, although the pathophysiological significance of altered UPS function in either pressure-overloaded cardiomyopathy or ischemia/reperfusion injury remains to be determined.

Cardiac proteasomal function decreases with ageing and may contribute to the temporal pathogenesis of some CHF. Although ubiquitination enzyme activity does not consistently change with age, accumulation of oxidized proteins and highly ubiquitinated proteins are associated with ageing and some ageing-related diseases, raising the possibility that the proteasome degradation pathway is impaired. Proteasome peptidase activities in cytosolic proteins extracted from the hearts of Fisher 344 rats decreased from 8 to 26 months, and this could be partially attributed to decreases in 20S proteasome abundance in the heart. The ability of cardiac 20S proteasomes purified from aged rats to degrade [14C]-methyl casein in vitro was also decreased significantly, compared with activity from younger rats. These decreases in proteolytic activity of senescent proteasomes are correlated with alterations in subunit composition and/or posttranslational modifications of the proteasomal subunits. Taken together, these lines of evidence are consistent with the hypothesis that the removal of abnormal proteins by the proteasome is insufficient in failing human hearts, and, in the future, it may be productive to examine the status of UPS proteolytic function in the heart during progression to heart failure.

However, the potential therapeutic manipulation of proteasomal activity is fraught with unknowns. Although acute and short-term proteasome inhibition with pharmacological inhibitors reduced myocardial ischemia/reperfusion injury, possibly through inducing heat-shock responses and preventing nuclear factor κB activation, pharmacological inhibition of 20S proteasomes caused necrotic and apoptotic cell death in cardiomyocyte cultures. Interestingly, a recent case report from a lung cancer patient suggests that chemotherapy using the proteasome inhibitor bortezomib causes reversible severe cardiac failure. In a separate report on relapsed and refractory multiple myeloma patients receiving a regimen containing bortezomib, heart failure is described as a cause of death unrelated to cancer progression. These observation and reports are sporadic and preliminary but raise the possibility that proteasome malfunction may be sufficient to cause heart failure in humans.

**Future Directions**

The importance of these basic cellular processes in normal cardiac function and the development of cardiovascular disease is clearly established. However, our understanding of the molecular details and relative importance of nascent protein folding, maturation, aggregation, and degradation in the healthy and diseased heart is in its infancy. PQC does appear to be compromised in a range of cardiac disease, as does chaperone and proteasome activity, and accumulation of presumably toxic PAOs in failing animal and human hearts is intriguing. Inadequate PQC can lead to the accumulation of misfolded and damaged proteins that, in turn, further impairs PQC in the heart, forming a vicious cycle that can lead to compromised cardiomyocyte function and cell death. Nevertheless, the necessity and sufficiency of these processes have, with rare exceptions, not been established in human cardio-
vascular disease. The impact of cardiomyocyte-restricted UPS inhibition on the development of heart failure should be addressable in animal models using gain- and loss-of-function studies restricted to the relevant cell types. Similar approaches can be used to address whether and how UPS malfunction causes heart failure, the role proteasome malfunction plays in the process, and whether acute or chronic manipulation of proteasomal activity is a viable therapeutic approach. The provocative parallels between the neurodegenerative and cardiovascular diseases, which can be drawn using the commonalities of protein conformation-based pathology, need to be explored in detail, and any potential therapeutics based on these processes evaluated in both systems.

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Disclosures
None.

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