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The Final Stage of Gene Expression: Chaperones and the Regulation of Protein Fate

John Ashkenas and Peter H. Byers

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Classically, geneticists are accustomed to thinking of missense mutations as relatively benign, or at least less hazardous than clear null alleles such as large genomic deletions or nonsense mutations. At the cellular level, however, the opposite is often the case; missense mutants that are synthesized but fail to mature along the normal folding pathway may prove more troublesome than simple loss of expression.

Cells possess a robust machinery to degrade mRNAs that, as a consequence of genetic lesions or errors in RNA processing, display premature-termination codons. The cues by which inappropriate breaks in open reading frames (ORFs) are recognized are uncertain (Maquat 1996)—even the subcellular location of nonsense-mediated decay is controversial—but the consequence is unambiguous: thanks to this monitoring process, many prematurely terminating alleles become true nulls. Experience with genetically altered mice confirms the wisdom of this regulatory strategy. Transgenic animals that express genes either ectopically or with abnormal sequences are often manifestly sick. On the other hand, gene targeting, which typically creates null alleles of the gene of interest, often leads to normal or nearly normal phenotypes, probably because of functional redundancy within the genome (Wang et al. 1996). In mice as well as humans, lack of gene expression may be more benign than abnormal gene expression.

No general mechanism exists, at the nucleic-acid level, to convert missense mutations to null alleles, but the cell is far from helpless to restrict the expression of abnormal proteins. The machinery that assists the folding of nascent proteins to their final native conformation also identifies unfolded or misfolded polypeptides. When things go right, this same system leads to the efficient removal of these abnormal proteins. Genetic disease, then, arises not solely because of a defect in a protein structure but, rather, because the machinery that disposes of aberrantly folded proteins is for some reason not equal to the task.

Address for correspondence and reprints: Dr. John Ashkenas, American Journal of Human Genetics, University of Wahington, Box 357470, Seattle, WA 98195-7470. E-mail: jayemay@u.washington.edu

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The Ubiquitin-Mediated Degradation Pathway and Its Targets

Protein degradation, the final stage in cellular regulation of gene expression, represents the last chance for a cell to suppress the potentially toxic effects of expression of aberrant proteins. One pathway that helps clear misfolded proteins is the degradative mechanism used in the turnover of naturally short-lived cytoplasmic proteins, the ubiquitin/proteasome pathway (Finley and Chau 1991), in which proteins are targeted for degradation by covalent modification. Ubiquitin, a small and highly conserved protein, is conjugated onto a lysine residue of a target protein by a ubiquitin-conjugating enzyme (UBC). Subsequently, more ubiquitin proteins are conjugated to the ubiquitin adduct, and this process can continue indefinitely to form a polyubiquitin chain. Polyubiquitinated proteins are recognized and rapidly degraded by a large multisubunit complex of proteinases, the 26S proteasome. Protein species that are marked for degradation can be identified on protein gels by their "ladder" of more slowly migrating polyubiquitinated forms. Because the proteasome will degrade most polyubiquitinated species, a crucial step in controlling protein turnover is the initial recognition of a target by a UBC. The determinants for this recognition have been defined in certain proteins that, in their wild-type form, degrade rapidly. In some cases, the exposed N-terminal residue plays a key role in determining protein longevity (Varshavsky 1992); in other cases, larger domains are required for rapid turnover (Rechtsteiner and Rogers 1996). The destabilizing determinants in misfolded proteins are not known, but the events downstream of UBC appear to be the same for both normal and misfolded targets.

Proteasomes are located in the cytoplasm and the nucleus, but neither they nor ubiquitin is found within the lumen of the secretory organelles. Hence, it was long assumed that turnover of unfolded or unassembled en-

doplasmic reticulum (ER) proteins must occur through an endogenous ER proteolytic pathway distinct from the lysosomal or proteosomal pathways. This view has shifted (Kopito 1997) with reports that degradation of ER-membrane proteins and even ER-lumenal proteins can be inhibited when the ubiquitin/proteasome pathway is compromised. Thus, in yeast, mutations that affect ubiquitin, UBC enzymes, or subunits of the 26S proteasome block degradation of secretory proteins (Hiller et al. 1996). Similarly, in mammalian cells, degradation of ER-associated secretory (Qu et al. 1996) or transmembrane (Jensen et al. 1995; Ward et al. 1995) proteins requires polyubiquitination and is sensitive to lactacystin, a proteasome inhibitor that is not known to inhibit any other proteinases. The process by which lumenal components are "delocated" (Kopito 1997) back to the cytoplasm from the ER remains unexplained, but chaperone proteins, as described below, may participate (Qu et al. 1996).

Components of the Protein-Folding Machinery

The heat-shock response (Leppa and Sistonen 1997) provided the first glimpse of a cellular machinery that could discriminate between native and non-native protein conformations. Heat shock–induced proteins (HSPs) mediate protein refolding to the native conformation after heat or some other environmental shock causes a cell to accumulate unfolded protein. HSPs, which fall into several families, use ATP energy to drive the refolding process. In addition to heat, such conditions as ischemia (Kuznetsov et al. 1996) or proteasome inhibition (Bush et al. 1997) cause the various HSPs to be induced in concert, and they result in cells becoming tolerant to similar, more severe treatments.

Even without stress to induce them, HSPs and other classes of molecular chaperones are expressed in healthy cells, where they act in protein biosynthesis to stabilize nascent protein chains and to unfold and refold proteins as they cross from the cytoplasm into membrane-bounded organelles. There is considerable evidence for specialization among chaperones, in their subcellular distribution, in their preferred substrates, and in their mode of interaction with substrates. Hsp104, for example, a chaperone in the yeast cytoplasm, serves to solubilize proteins from heat shock–induced protein aggregates but not to prevent aggregation (Parsell et al. 1994). Likewise, Lhs1p, an Hsp70 homologue found in the yeast ER, appears not to be involved in biosynthetic folding pathways in the secretory pathway but, rather, to permit disaggregation and refolding of several proteins after heat-induced denaturation (Saris et al. 1997). Hsp47, several groups have argued, specifically mediates the folding of collagen triple helices in the ER (Nagata 1996). Despite their functional diversity, the various HSP homologues probably operate on their protein substrates by common mechanisms.

Protein Folding in the Secretory Pathway

Many of the resident proteins of the ER assist in the maturation of secretory proteins destined for other compartments or for the extracellular space. They mediate insertion through the ER membrane (Rapoport et al. 1996), they carry out posttranslational modifications such as glycosylation, proteolytic trimming, and disulfide bond formation, and they assist in folding and assembly of secreted and integral membrane proteins (Hebert et al. 1995*a*, 1995*b*). Three of the major chaperones of the ER are BiP, a member of the Hsp70 family; calreticulin, a lumenal protein; and calnexin, a transmembrane protein. The last two proteins behave as lectins—they bind to sugar moieties on nascent glycoproteins (Ware et al. 1995)—but it appears that they can also bind to nascent proteins that lack carbohydrates (Kim and Arvan 1995).

The induction of the ER-resident HSPs is regulated, along with cytoplasmic and mitochondrial HSPs, by the transcriptional activator, heat-shock factor, but recent work has identified in yeast an independent pathway that specifically induces ER-resident chaperones when unfolded proteins accumulate in this organelle. This unfolded-protein response (UPR) pathway (fig. 1) is mediated by an ER-resident transmembrane receptor kinase protein Ire1p, which directly or indirectly senses the presence of unfolded protein in the ER lumen. Ire1p then transduces a signal that activates transcription of the yeast BiP homologue by altering the pattern of splicing of the mRNA encoding the transcription factor Hac1p (Cox and Walter 1996). This alternative splicing event (Sidrauski et al. 1996), which is novel and mechanistically unrelated to the process discussed by Cooper and Mattox (1997 [in this issue]), causes a change in the 3' end of the Hac1p ORF. Although both the induced and the uninduced spliced forms of Hac1 mRNA encode active transcription factors, the uninduced protein is efficiently degraded, probably by the ubiquitin/proteasome pathway (Cox and Walter 1996). Activation of Ire1p by unfolded proteins in the ER also activates another transcription factor, Gcn5p, which interacts directly with Ire1p. Welihinda et al. (1997) argue that Gcn5p and Hac1p form part of a large transactivator complex that induces ER chaperones but not cytoplasmic heat-shock proteins. The UPR pathway has so far only been demonstrated in yeast, but it will be of great interest to learn to what extent it is conserved in human cells.

Chaperones as Defense Attorney, Judge, and Executioner

Chaperones are present at millimolar levels in the ER lumen, in considerable excess over substrates, and, in

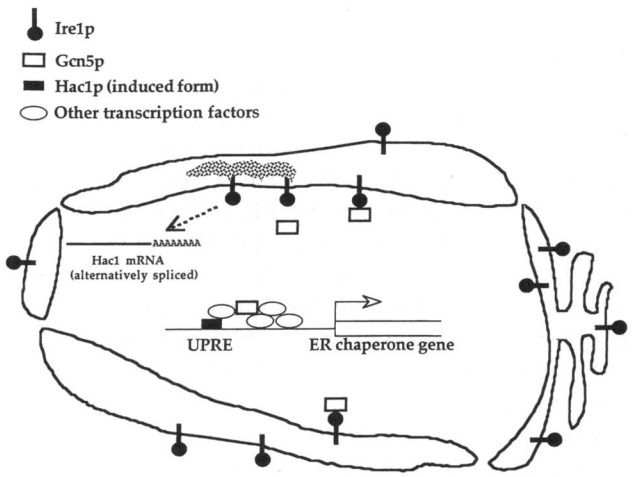

Figure 1 UPR. Two pathways in the transcriptional induction of ER chaperones have been described. The transmembrane protein Ire1p signals the presence of accumulated unfolded proteins in the ER. Activated Ire1p, shown here bound to a cloud of unfolded protein at its lumenal aspect, transmits a signal that results in the alternative splicing of the Hac1 mRNA. As a result, the induced form of the Hac1 protein is synthesized, and this protein binds to the UPR element (UPRE) in the promoter of various ER chaperone genes (Cox and Walter 1996). A second pathway, which may be independent of the first, involves the activation of the Gcn5 protein. This protein binds directly to Ire1p and participates in a complex of DNA-binding proteins that activates transcription of the yeast BiP homologue (Welihinda et al. 1997). Most likely, Gcn5p is released from Ire1p as this receptor is activated by unfolded proteins. Ire1p is shown here signaling directly into the nucleoplasm, but it is also possible that it faces the cytoplasm and that the activated signaling molecules need to pass into the nucleus to act as transcriptional regulators.

addition to interacting with nascent secretory proteins, they participate in numerous weak interactions with other ER-resident proteins (Hammond and Helenius 1995; Hebert et al. 1995*b*). These interactions are presumed to give the ER lumen some viscosity and internal structure (Kuznetsov et al. 1997): Helenius and colleagues compare it to a mixed-bed affinity-chromatography resin through which a secretory protein needs to pass as it interacts with the different resident proteins at different stages of the maturation pathway. Indeed, Kim and Arvan (1995) have shown that the folding pathway of one secreted glycoprotein, thyroglobulin, proceeds through intermediate stages, first interacting with calnexin and later dissociating from it and binding BiP.

To generate a quality-control system from the interactions between the nascent protein and the chaperones, there needs to be at least one step in which chaperones or other proteins distinguish between properly and improperly folded proteins, release the mature protein, and either retain the unfolded forms or target them for degradation. Although this crucial recognition step is not fully understood in any instance, Helenius and colleagues have proposed a simple model based on sequential rounds of glucosylation and deglucolsylation of N-linked sugars on glycoproteins (fig. 2) (Hammond and Helenius 1995; Hebert et al. 1995*a*, 1995*b*). N-linked oligosaccharides are added cotranslationally to proteins as they enter the ER, and they are trimmed by glucosidases to the monoglucosylated form. This sugar structure mediates binding to calnexin and/or calreticulin, and, according to the model, the protein will remain bound until released in deglucosylated form by a glucosidase. The key quality-control step, the readdition of a glucose, is then mediated by a glucosyltransferase that acts specifically on unfolded glycoproteins. The reglucosylated protein is then once again retained on calnexin or calreticulin; folded glycoproteins, which cannot be reglucosylated, are free to leave the ER and continue to move through the secretory pathway. This mechanism may apply generally to nascent secretory proteins with N-linked carbohydrates and may provide a basis for understanding other classes of quality-control events.

Such a mechanism can be extended to explain the turnover of misfolded proteins that are retained by chaperones. A simple model suggests that proteins bound to chaperones are safe from proteolysis (as are most properly folded proteins) and that rounds of binding and release from chaperones work toward protein maturation (Farr et al. 1997). Folded proteins in the ER are released to traverse the secretory pathway, but retained unfolded proteins are captured by the degradative ma-

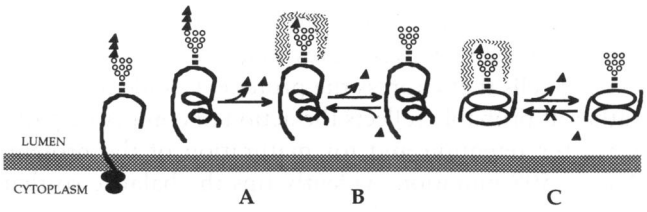

Figure 2 Model for the selective retention of unfolded glyoproteins in the ER (Hebert et al. 1995*b*). As a nascent protein (*curved line*) is extruded across the ER membrane (*shaded bar*), an oligosaccharide chain (shown here above the curve of the protein) is added cotranslationally. A newly synthesized glycoprotein is then released in unfolded form and, in step A, its oligosaccharide chain is enzymatically trimmed by glucosidases that remove terminal glucose units (*triangles*). Once the oligosaccharide has been trimmed to the monoglucoslylated form, chaperone proteins (*shaded figure*), such as calnexin or calreticulin, may bind the glycoprotein. The glycoprotein may be released from the chaperone if the final glucose is removed by a glucosidase (*step B, rightward-pointing arrow*), but a specific glucosyltransferase, acting on the unfolded protein, can replace the lost glucose unit so that the monoglucosylated protein can bind the chaperone once again (*step B, leftward-pointing arrow*). However, once the protein is folded, this process becomes irreversible: the glucosidase can act on the folded glycoprotein and release it (*step C, rightward-pointing arrow*), but the glucosyltransferase cannot act on the folded protein, and the folded glycoprotein is free to continue through the secretory pathway.

chinery. Such capture could be a purely passive and stochastic process, but it appears that, at least in some cases, chaperones play an active role in protein degradation. Hayes and Dice (1996) cite evidence that the Hsp70 homologue Hsc73 is required on both the cytoplasmic side and the lumenal side of lysosomes in order to get efficient import of certain cytosolic proteins into this degradative organelle. In vitro, Hsc73 also mediates the degradation of unfolded protein by the cytoplasmic-ubiquitin/proteasome pathway. Furthermore, as discussed below, the transmembrane ER chaperone calnexin serves both to retain nascent glycoproteins and to target their abnormal variants for destruction.

Mutant Proteins in Human Disease

Human genetic disorders, whether dominantly or recessively inherited, commonly arise from sequence changes that alter protein function, such as enzymatic activity or structural or regulatory interactions. To the extent that the cell can recognize an altered protein, the protein can be redirected to the degradative pathway, and the cell can be spared the consequences of accumulating the abnormal protein.

In the case of the cystic fibrosis transmembrane conductance regulator (CFTR), a multipass integral membrane protein, it has been shown that a number of subtle mutations, including the common disease allele ΔF508, cause CFTR to persist in the ER (Cheng et al. 1990). In fact, ~75% of all wild-type CFTR protein fails to exit the ER but accumulates there and eventually is degraded by the ubiquitin/proteasome pathway (Jensen et al. 1995; Ward et al. 1995). This inefficient maturation is not an artifact of overexpression, since it is observed even in cells that express the protein at moderate levels; rather, it probably reflects the action of competing pathways for retention and for maturation of the protein. The ΔF508 mutation evidently tips the balance so that the protein is retained quantitatively, but, despite its different fate in biosynthesis, this mutation does not grossly perturb the structure of the protein or alter its activity as an ion channel. When assayed in the ER, wild-type and ΔF508 CFTR act as chloride channels with indistinguishable kinetic properties (Pasyk and Foskett 1995), indicating that the mutant protein folds to a close approximation of its native conformation. Hence, at least for those who are homozygous for this common mutant allele, cystic fibrosis appears to be not so much a disorder of the CFTR as of the protein-maturation machinery that fails to release it as a functional protein to the cell surface. This raises the possibility that differences in chaperone function could modulate the severity of the symptoms of the disease, and it may explain the rare individuals homozygous for this allele who are only minimally symptomatic.

Although the role of chaperones in cystic fibrosis remains speculative, the spectrum of symptoms in another heritable disorder, α1-antitrypsin (α1-AT) deficiency, appears to reflect variability in the cell's response to abnormal proteins. Even as part of its normal folding pathway (Ou et al. 1993; Wu et al. 1994), α1-AT interacts with the transmembrane ER chaperone calnexin, but only the mutant protein accumulates in the ER and is degraded by the proteasome. In this degradative pathway, calnexin itself is polyubiquitinated, presumably on its cytoplasmic face (Qu et al. 1996). This finding suggest that the binding of mutant α1-AT to calnexin causes a conformational change in the chaperone, which is recognized by cytoplasmic UBCs. The ubiquitination of calnexin appears to mark the whole complex for transport from the ER and for degradation by the proteasome.

Mutations in the α1-AT gene have distinct consequences for liver—which expresses this proteinase inhibitor—and for other tissues that are affected by its absence (Wu et al. 1994; Teckman et al. 1996). Loss of secreted α1-AT, as seen in people homozygous for the missense PiZ or PiS alleles, alters the balance of elastase and inhibitors in the lung and so predisposes to emphysema. Whereas PiS homozygotes are free of severe liver symptoms, a subset of PiZ-homozygous individuals, described as "susceptible hosts," develop cirrhosis and other liver disorders as abnormal protein accumulates in the distended ER of hepatocytes Other individuals with the PiZ-homozygous genotype, "protected hosts," degrade the mutant protein efficiently, and their liver cells are morphologically normal. Wu et al. (1994) expressed normal and PiZ α1-AT proteins in fibroblasts from protected and susceptible individuals and observed this difference in efficiency of degradation in these cells as well. Furthermore, the two mutant α1-AT alleles behaved consistently in the cell-culture system: Whether the cells used are from individuals susceptible to or protected from liver disease, both classes of abnormal proteins are retained in the ER. When expressed in susceptible cells, there is a lag in the degradation of the retained protein, and the more extreme lag was associated with the more severe PiZ allele (Teckman and Perlmutter 1996). Teckman et al. (1996) have begun to define the biochemical features associated with susceptibility. In cells from one susceptible host, they find that abnormal α1-AT interacts poorly with calnexin; in other susceptible hosts, the protein is degraded poorly even though its interaction with calnexin is efficient. These results strongly suggest that the susceptibility phenotype reflects genetic differences in the proteolysis of the mutant proteins, possibly in the interaction between the mutant protein and calnexin.

Like α1-AT, collagens are secreted through the ER, and, in some cases, mutations cause massive accumulation that is apparent by examination of affected tissues

(e.g., see Smith et al. 1997). It is likely that mutations in different functional domains of collagen-precursor chains activate an array of cellular strategies to mitigate the damage that could result if these molecules were secreted. Mutations in the carboxyl-terminal propeptide—the domain through which the initial chain-chain interactions occur—interfere with but do not abolish assembly of collagen trimers. Such mutations induce BiP, possibly through the UPR pathway, and lead to moderately efficient degradation of the abnormal proteins (Chessler and Byers 1993; Chessler et al. 1993). However, mutations in the triple-helical domain that interfere with helix propagation result in ER retention with very slow degradation and do not activate the UPR, even though the triple helix of these molecules is not "folded" at the normal temperatures (Chessler and Byers 1992). Instead, retention is mediated by interaction with the ER-resident protein, prolyl hydroxylase, which binds nonfolded helical structures that are substrates for hydroxylation. The mechanism by which the slow degradation of ER-retained molecules is a consequence of binding to prolyl hydroxidase is uncertain; it is likely that at least two degradative pathways exist for these molecules (Lamandé et al. 1995).

The ER is not the final point of defense against missense mutations for secreted proteins. Such proteins, collagens included, often form a complex array that involves multiple interactions with other proteins in the extracellular matrix (ECM). Collagen-fibril formation requires that the shape of the molecule be maintained, and any change in the triple-helical domain interferes with aggregation of those molecules into fibrils. Hence, fibril formation tends to exclude abnormally formed collagen triple helices, which appear to be degraded by ECM proteinases—another device to suppress expression of a potentially harmful protein. Abnormal collagen molecules that evade this system and are integrated, even at low levels, can compromise mineralization or other functions of the fibril.

Quality-control mechanisms, operating at multiple sites within a cell and even in the extracellular space, remove abnormal proteins and prevent damage to cells and tissues. Although they can be remarkably effective, they may be less efficient than the analogous process that targets truncated mRNA, in part because their substrates have been amplified by translation and, in part, because the substrates are more heterogeneous. The emerging hints of genetic diversity in this system reaffirm the need for geneticists to understand the mechanisms of gene regulation at all levels, because these provide the biological context in which genetic variation plays out to create disease.

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