The Role of the Ubiquitin Proteasome Pathway in Keratin Intermediate Filament Protein Degradation

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Lung injury, whether caused by hypoxic or mechanical stresses, elicits a variety of responses at the cellular level. Alveolar epithelial cells respond and adapt to such injurious stimuli by reorganizing the cellular cytoskeleton, mainly accomplished through modification of the intermediate filament (IF) network. The structural and mechanical integrity in epithelial cells is maintained through this adaptive reorganization response. Keratin, the predominant IF expressed in epithelial cells, displays highly dynamic properties in response to injury, sometimes in the form of degradation of the keratin IF network. Post-translational modification, such as phosphorylation, targets keratin proteins for degradation in these circumstances. As with other structural and regulatory proteins, turnover of keratin is regulated by the ubiquitin (Ub)-proteasome pathway. The degradation process begins with activation of Ub by the Ub-activating enzyme (E1), followed by the exchange of Ub to the Ub-conjugating enzyme (E2). E2 shuttles the Ub molecule to the substrate-specific Ub ligase (E3), which then delivers the Ub to the substrate protein, thereby targeting it for degradation. In some cases of injury and IF-related disease, aggresomes form in epithelial cells. The mechanisms that regulate aggresome formation are currently unknown, although proteasome overload may play a role. Therefore, a more complete understanding of keratin degradation—causes, mechanisms, and consequences—will allow for a greater understanding of epithelial cell biology and lung pathology alike.

Keywords: intermediate filaments; ubiquitin proteasome pathway; degradation

Proteins are continually being hydrolyzed to their constituent amino acids by highly selective proteolytic systems. Interestingly, proteins are degraded at widely differing rates that can vary from minutes for some regulatory enzymes, to days or weeks for proteins such as actin and myosin in skeletal muscle, or months for hemoglobin in the red cell. This process of continually destroying cellular proteins has important homeostatic functions, such as regulating cell cycle, signal transduction, differentiation, and response to stress. In all tissues, the majority of intracellular proteins are degraded by the ubiquitin (Ub)-proteasome pathway (UPP). If the UPP fails to degrade intracellular proteins, such as during stress, the accumulation of misfolded protein may overload the proteasome, potentially leading to pathogenesis.

PROTEIN DEGRADATION VIA THE UPP

The degradation of a protein via the UPP involves two successive steps: (1) tagging of the substrate by covalent

attachment of multiple Ub molecules, and (2) degradation of the tagged protein into small peptides by the 26S proteasome complex with release of free and reusable Ub (Figure 1). The E1 (Ub-activating enzyme) and E2s (Ub-carrier or conjugating proteins) prepare Ub for conjugation, but the key enzyme in the process is the E3 (Ub-protein ligase), because it recognizes a specific protein substrate and catalyzes the transfer of activated Ub to the target protein. The 2004 Nobel Prize in Chemistry was awarded to Aaron Ciechanover, Avram Hershko, and Irwin Rose (<http://nobelprize.org/chemistry/laureates/2004/>) for the discovery of the UPP.

Ub and the Ligases

Ub is composed of 76 amino acids. A key glycine residue within the C-terminus is required for its conjugation to other Ub molecules and target substrates; it also contains internal lysine residues that are required for the formation of polyubiquitin chains. The initial step in conjugation of Ub onto proteins is activation of Ub at its C-terminus by the Ub-activating enzyme E1. This abundant 110-kD enzyme uses ATP to generate a Ub thioester, a highly reactive form of Ub (1, 2). Once activated, the Ub that is bound to E1 via the thioester linkage is transferred to a sulfhydryl group of one of the Ub carrier proteins or an E2. The E2s are small proteins that share a conserved 16-kD core containing the cysteine that forms a thioester linkage with the activated Ub.

The more than 1,000 E3 ligases in cells confer high specificity to the UPP. E3 ligases function either as single proteins or in complexes to link E2s to proteins either directly or via another protein (2). Essentially, E3s act as scaffolds and often catalyze the transfer of the activated Ub from E2s to a lysine in the target protein and subsequently to lysines that are present in Ub, yielding a substrate-anchored chain of Ub molecules. The two best-studied classes of E3s that may play predominate roles in keratin intermediate filament (IF) protein degradation are the HECT (homologous to E6-AP carboxy-terminus) domain proteins and the RING finger proteins. HECT-domain proteins are large monomeric E3s possessing a homolog C-terminal domain, which accepts the activated Ub from the E2. This exchange occurs via the formation of a thioester linkage with Ub, catalyzing Ub transfer to the substrate. Unlike HECT-domain-containing E3s, E3s with RING finger domains are either monomeric enzymes or multi-subunit complexes and do not form thioester bonds with Ub. Rather, they typically serve as scaffolds that bring the substrate and the E2 into close proximity and may assist in formation of a Ub chain (3). Monomeric RING finger E3s include the oncoprotein Mdm2 (4), a physiologic regulator of p53 stability in normal cells, and c-Cbl (5), which catalyzes ubiquitination of certain cell surface receptors.

The 26S Proteasome

Found both in the cytosol and nucleus of cells, the 26S proteasome catalyzes the rapid degradation of ubiquitinated proteins (6). This enormous complex is composed of a central barrel-shaped 20S core particle with a 19S regulatory particle at

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Figure 1. Protein degradation via the ubiquitin proteasome pathway.

either or both of its ends (7). The proteolytic capacity of the 26S proteasome lies within the 20S core. It is composed of four stacked, hollow rings, each containing seven distinct but related subunits (8). The two outer α rings are identical, as are the two inner β rings. Three subunits of the β rings contain the proteolytically active sites, positioned on the interior face of the cylinder. The outer α subunits of the 20S particle surround a narrow, central, and gated pore through which substrates enter and products exit (8). The physical architecture of the 19S particle selects, prepares, and translocates substrates into the 20S core for degradation. The outer lid of the 19S particle contains subunits that bind polyubiquitin chains, as well as two deubiquitinating enzymes (also called isopeptidases) that disassemble Ub chains, allowing for reuse of Ub in the degradation of other proteins (9). After deubiquitination, target proteins are unfolded and processed through the 20S particle in an ATP-dependent manner. After the substrate enters the central chamber of the 20S particle, the polypeptide is cleaved by the six proteolytic sites on the inner face of the chamber, resulting in small peptides that range from 3 to 23 residues in length (10). These peptides are then rapidly digested into constituent amino acids by the abundant cytosolic endopeptidases and aminopeptidases and reused to synthesize new proteins or are metabolized (11).

STRUCTURE, FUNCTION, AND DEGRADATION OF IFs

IFs, microfilaments (MFs), and microtubules (MTs) are the key structural proteins that form a dynamic cytoskeleton. IFs make up a large protein family that includes 73 unique gene products, which places the genes encoding them among the 100 largest gene families in humans (12). Several features distinguish IFs from MFs and MTs, including IF structural diversity, tissue- and cell-selective expression, unique subcellular compartment distribution (lamins are found in the nucleus, whereas the remaining IFs are in the cytoplasm), and, most relevant, their involvement in more than 80 human diseases (Human Intermediate Filament Database, <http://www.interfil.org/index.php>)

Figure 2. Structure of intermediate filaments.

(13). Another contrast to MFs and MTs is the fact that, to this date, no inhibitors of IF assembly are known. IFs are grouped into six types (types I–VI): types I through IV are found in the cytoplasm, the type V IF proteins are found in the nucleus, and those classified as type VI are found exclusively in the lens. Type III proteins, such as vimentin, are found primarily in endothelial, hematopoietic, and mesenchymal cells. The largest group consists of type I and type II keratins found in epithelial cells. In the cells of higher organisms, several types of IFs are expressed at once; for example, both type V lamins and type III vimentin can be expressed in parallel within a single cell type. Despite the great number of genes and diverse tissue distribution, the IFs in many different cell types are morphologically similar. They have a structurally conserved central rod domain, composed of heptad repeats that are required for the coiled-coil interactions involved in the assembly of IFs (14). The rod domain is flanked by variable N- and C-terminal domains. The basic unit of all IFs is a dimer composed of two α -helical chains that are oriented in parallel and interact to form a very stable coiled-coil rod (Figure 2). Dimers associate in an antiparallel, half-staggered manner to form a larger unit, which in turn grows in length to form protofilaments. Lateral associations of protofilaments give rise to protofibrils and three to four of these protofibrils intertwine to form the stable 10-nm filament. In epithelial cells, a single type I (acidic) and type II (neutral-basic) keratin chain form heterodimers. Alveolar epithelial cells specifically express four keratin proteins: K8, K18,

Figure 3. The keratin intermediate filament network is known to colocalize with ubiquitin in response to stress.

K7, and K19. In the lung, K8 and K18 are equally expressed, and we focus on these two proteins in this review.

Although the classical concept states that IFs are static structures merely serving a structural function within cells, a great deal of recent data support the idea that IFs are actually dynamic and important for many cellular functions. This paradigm shift occurred in part because studies using fluorescent recovery after bleaching and microinjection of fluorescently tagged IF proteins showed that a dynamic equilibrium exists between IF subunits (particles and squiggles) and IF filaments. In terms of IF function, it has been accepted for many years that IFs provide mechanical support to cells. We are now aware of many more IF functions, including their involvement in cellular migration, mitosis, and signal transduction. These functions are likely made possible by the dynamic nature of IFs, with the disassembly and reassembly processes dependent on reorganization. For example, IF assembly occurs sequentially from particles to squiggles to filaments in spreading cells (15, 16), and IFs disassemble during cell division. IF proteins can move rapidly in either a retrograde or anterograde direction along microtubule tracks, suggesting that cells possess a mechanism for regulating IF distribution to locally tune cellular properties, such as shape and adhesion. Vimentin IFs may organize to form a “cage” or shield at the cell periphery, presumably to enable cells (e.g., lymphocytes) to resist mechanical stresses, or they may rapidly reassemble to a perinuclear location during chemotaxis. Likewise, epithelial cells undergo a process to enable migration into injured areas of the alveolar epithelium. These changes are made possible through site-specific phosphorylation. Accumulating data suggest that epithelial cells’ keratin IFs can undergo rapid disassembly and degradation in response to insults such as mechanical stress, shear stress, or hypoxia. These data and other data suggest that the IF cytoskeleton transmits signals from the cell surface to all regions of the cytoplasm (17). IFs within the cytoplasm can transmit extracellular signals by binding to and regulating the activity of signaling proteins, either via a receptor or adaptor mechanism. Changes in IFs, occurring either through post-translational modification, composition, or association with other proteins, can modulate these signals. This signaling function regulates a variety of cellular programs, including stress response and migration, mitosis, differentiation, and more.

During many of these normal programs, such as mitosis, IFs undergo significant and reversible disassembly. In other cases, such as during stress, IFs may undergo irreversible protein degradation. Destruction of a protein can lead to a complete, rapid, and sustained termination of the process involving the protein as well as a change in cell composition. The rapid degradation of specific proteins permits adaptation to new physiologic conditions. At times this process can be detrimental to the function of cells, and in some cases it may actually be protective. For the most part, IF protein degradation is complete, but in some cases accumulation may occur. Accumulation of partially degraded IF protein can lead to a specific pathological feature known as aggresomes.

KERATIN IFs AND AGGRESOMES

The expression of IF proteins is highly regulated during development and cell differentiation, and can be markedly altered in cells and tissues undergoing an injury response. A diversity of human diseases is associated with severe alterations of IFs (18). One common pathological feature of many IF-related diseases is the accumulation of intracytoplasmic inclusion bodies or aggresomes consisting of modified IF proteins. Examples include the neurodegenerative diseases amyotrophic lateral scler-

osis, Pick disease, and Parkinson disease (19). Common to all these diseases are aggresomes made up mainly of IFs and Ub. In alcoholic hepatitis and other liver disorders aggresomes are referred to as Mallory-Denk bodies (MDBs) (20). The morphology and biochemical composition of MDBs in liver disorders is constant. MDBs are typically irregularly shaped, at times globular, and located near the nucleus. However, MDBs have also been observed at the cell periphery, associated in distinct cases with keratin IFs or desmosomes (21). These different locations were correlated to stages of MDB formation in the griseofulvin-fed mouse model; in this model, smaller, globular aggresomes reflect early formation of MDBs, whereas in later stages of formation, the MDBs become larger, perinuclear structures (22). Aggresomes are also observed in renal cell carcinoma (23), nonneoplastic pancreatic cells (24), and patients with interstitial pneumonitis (25), as well as in the cytoplasm of especially swollen alveolar epithelial cells. These cytoplasmic aggresomes are composed of intermediate-type fine fibrils, which are positive for anti-keratin antibody—predominantly K8 and variable amounts of K18 assembled in a nonfilamentous manner (26). In addition to keratin, nonkeratin components, such as Ub, have also been identified as constituents of aggresomes (19). These facts suggest that they represent a kind of pathological keratinization of injured epithelial cells, including those found in the alveolar epithelium of the lung.

Aggresomes consist of filamentous aggregates of misfolded proteins and other protein-based components to various degrees and combinations. The major constituents include: cyto-keratins (often post-translationally modified); chaperones, such as heat shock proteins (hsp 70, hsp 90); degradation machinery, including p62 and Ub; and a small group of other proteins (27, 28). Although aggresomes were first described in hepatocytes in 1911, it was not until 1979 that keratin IFs were identified as the major constituent (29). In epithelial cells, the main constituents are keratins 8 and 18 (K8 and K18), typically in a hyperphosphorylated state (30–32). In fact, hyperphosphorylated keratin IFs are plentiful in aggresomes of other cells, possibly even initiating their formation (31, 33). Signaling pathways, such as src, AKT, p38, and ERK, likely mediate this process (33–35). For example, p38 has directly been shown to phosphorylate K8 at Ser73 (34, 36). A study using diethyl-1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate (DDC)-primed mice showed that MAPK p38, Ub, and p62 colocalized with K8 in cytokeratin aggresomes (34). Inhibition of p38 phosphorylation by SB202190 prevented cytokeratin aggresome formation. Thus, hyperphosphorylation of K8 by p38 kinase seems to be a key step in the process of aggresome formation. These studies support the concept that keratin IF phosphorylation appears to act as a degradation marker for UPP, and that there is a connection between polyubiquitinated aggregates and proteasome overload. Keratin aggregation and aggresome formation has also been observed in human liver cells when the proteasome is inhibited by oxidative stress induced with ethanol treatment (37). The proteasome inhibitor, PS-341, induces formation of aggresomes, again characterized by colocalization of K8 and Ub in a perinuclear location (38). Another study using an *in vitro* kidney model showed that either overexpression or inhibition of proteasome activity allows formation of misfolded protein (cystic fibrosis transmembrane conductance regulator) aggregates to occur (39).

Although the formation of aggresomes arises by multiple mechanisms, their morphology and biochemical composition remain fairly constant across tissue type and pathology. The location of these bodies within the cell, on the other hand, appears to be less consistent. For example, although aggresomes are usually found near the nucleus, they may also be present throughout the cytoplasm or near the cell periphery associated

with desmosomes (21, 40, 41). In general, the aggregates appear to sequester into one or more foci throughout the cell. Morphologically, the basic filamentous structure can vary from a completely random arrangement to filaments oriented parallel to one another. The IFs contained within Mallory bodies (aggresomes) was described by Franke and colleagues in 1979 as “randomly oriented, branched filaments” with diameter 14 to 20 nm, much thicker than the 10-nm width of normal IFs (29). Also unlike normal keratin IFs, these proteins were covered by a thick coat of threads projecting out from the filament surface. Other characteristics of aggregates include poor solubility, abnormal localization, and nonnative secondary structure.

KERATIN IFs AND THE UPP IN ALVEOLAR EPITHELIAL CELLS

The keratin IF network in alveolar epithelial cells have different measured responses depending on the external stimuli. For example, alveolar epithelial cells exposed to stretch at 20% deformation show no change in keratin IF assembly state, suggesting that stretch does not activate signal transduction pathways involved in their disassembly and degradation (30). In contrast, shear stress appears to promote the disassembly and reorganization of the keratin IF network in alveolar epithelial cells in a time-dependent manner (37, 38). Brief periods of shear stress cause the keratin IF network to reorganize from thin, long, and relatively straight filaments into thick, wavy, “tonofibrils” (keratin IF bundles). In conjunction with this reorganization, keratin protein solubility decreases and the exchange rate of keratin subunits significantly increases. Importantly, the stiffness of these cells also increases by approximately 40%, with local stiffness values decreasing according to distance from the nucleus. The increase in stiffness enables cells to withstand injurious mechanical forces (e.g., mechanical ventilation), whereas the keratin network distribution would protect the nucleus and enhance cellular plasticity at the cell periphery. The rate and extent of keratin IF disassembly and degradation exhibits a dependence on the degree of shear stress (30); increased levels of shear stress accelerate the rate of disassembly and reorganization of the keratin IF network. The disassembly and degradation of keratin proteins is regulated by post-translational modifications, mainly phosphorylation and ubiquitination (24, 26). The initial step in this process appears to be “tagging” the keratin protein for degradation via phosphorylation. Phosphorylation is known to occur within the head and tail domains, which are responsible for most of the structural heterogeneity and presumed tissue-specific functions of IF (42). In the case of keratin 8, a number of *in vivo* phosphorylation sites have been mapped, and these play essential roles in regulating filament assembly and disassembly *in vivo* (42). IF can be phosphorylated by a variety of kinases, including protein kinase C, which is activated by mechanical forces. As such, shear stress, but not stretch, activates PKC δ and phosphorylates K8 on serine 73, which then initiates the disassembly and degradation of keratin IF in alveolar epithelial cells (30, 32).

The keratin IF network is known to colocalize with Ub in response to stress (Figure 3) (32). In our study, continuous exposure to shear stress resulted in the formation of perinuclear aggresomes containing both keratin and Ub. The aggresomes were present in approximately 30% of the cells. Colocalization of Ub cross-reactive protein with the keratin IF network has been observed in A549 cells as well (43). These structures may form when the proteasome capacity is exceeded (39) or inhibited, resulting in the subsequent accumulation of ubiquitinated proteins within aggresomes. Jaitovich and colleagues demonstrated that soluble keratin protein is degraded at an

accelerated rate compared with insoluble, filamentous keratin protein (32). These results demonstrated that insoluble, filamentous keratin protein is not efficiently degraded via the UPP compared with soluble, nonfilamentous keratin. This could be due the restrictive pore size of the 19S proteasome complex which requires nano-sized proteins to be “fed” through the proteasome. Proteasome inhibition by MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) prevents degradation of keratin IF network in response to mechanical strain (32). Other studies similarly report an accumulation of phosphorylated K8 and K18 when the proteasome is inhibited (44).

Different E2 enzymes, such as UbcH5b, UbcH5c, and Ubc6, are required for the shear stress- and/or hypoxia-mediated Ub-proteasome degradation of the keratin proteins (32). Ubc5 family members and Ubc3 participate in the degradation of keratin IF proteins under mechanical stress (32), whereas Ubc6 mediates the keratin degradation under hypoxia (45). Members of the UbcH5 family are highly homologous. UbcH5b and c are 97% identical at the amino acid level (with a difference of only four amino acids), and it is likely that they have similar biologic activities (46). The various E2s associated with keratin IF protein degradation may reflect the different cellular functions associated with Ub E2s. Although the precise biological role of many of the Ub E2s is not well defined in mammalian cells, there is accumulating evidence that E2s have unique functions. For example, Ubc3 is required for the G1-S transition, Ubc2 is central to DNA repair, and Ubc 4/5 is required for viability in *Saccharomyces cerevisiae* (1). In the case of hypoxic stress, overexpression of Ubc-6–dominant negatives prevent keratin degradation. Ubc-6 is an integral membrane protein that has been previously described to be localized to the endoplasmic reticulum and to the nuclear membrane, and to mediate the turnover of the short-lived transcriptional regulators and other endoplasmic reticulum-associated protein (47). These functions may be dependent on the specificity for the individual E3s. The E3 protein associated with keratin IF protein degradation has not yet been identified.

CONCLUSIONS

It is unclear whether accumulation of proteins such as keratin IFs plays a correlative, or causative, role in lung injury or epithelial-related disease. Formation of aggregates consisting of modified IF, Ub, p62, chaperones, and other proteins most probably occurs in response to cellular malfunction or stress, but the question then is whether their presence is protective, harmful, or neutral to injured or diseased cells. On the one hand, sequestration of abnormal proteins should promote cellular homeostasis by preventing their interference with important cellular processes. Cells containing aggresomes are typically viable (48), and aggresome (Mallory body) formation is even reversible and accompanied by the reappearance of the keratin cytoskeleton in mouse hepatocytes (41). On the other hand, these aggregates could be toxic to cells by interfering with the mechanical functions of a cell or by hindering the function of degradation machinery. In addition, aggregation of ubiquitinated proteins has been shown to impair the function of the UPP, resulting in cell death (49). Finally, the withdrawal of cytoplasmic components, such as the IF cytoskeleton, has tremendous implications for epithelial cellular function and, at the larger scale, tissue integrity. The most striking evidence for this fact can be seen in blistering diseases, such as epidermolysis bullosa simplex (EBS), caused by point mutations of keratins, wherein the mechanical integrity of cells is critically compromised (18, 50). Identification of the components involved in the Ub-proteasome-mediated degradation of keratin IFs will be essen-

tial in understanding how these key structural proteins may contribute to the physiologic functions of alveolar epithelial cells. Whether or not these processes are coregulated has great implications for understanding the formation and accumulation of IF aggresomes.

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