Immunologic Aspects of Protein Degradation by the Ubiquitin-Proteasome System

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Abstract

The ubiquitin-proteasome pathway has a central role in selective degradation of intracellular proteins. Among the key proteins degraded by the system are those involved in the control of inflammation, cell cycle regulation and gene expression. With numerous important cellular pathways affected, derangements in the ubiquitin system were shown to result in a variety of human diseases including malignancies, neurodegenerative diseases and hereditary syndromes, and proteasome inhibition was implicated as a potential treatment for cancer and inflammatory conditions. Two proteasome inhibitors are currently under clinical evaluation for multiple myeloma and acute ischemic stroke. The ubiquitin system also has an important function in the immune and inflammatory response. It is involved in antigen processing and presentation to cytotoxic T cells, and the activation of nuclear factor-kappa B – the central transcription factor of the immune system. Since the proteasome is the central source of antigenic peptides that are presented to the immune system, some viruses, such as the Epstein-Barr virus, developed escape mechanisms that manipulate the ubiquitin-proteasome system in order to persist in the infected host. Understanding the mechanisms underlying the production of viral antigens by the ubiquitin-proteasome system may have therapeutic applications such as future development of vaccines.

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Cells must be able to respond to rapid changes in both their internal and external environments. A particularly sensitive, rapid and reversible response to environmental stimuli or to a change in cell state is the post-translational modification of specific proteins. Small molecule modifications, such as phosphorylation and acetylation, are well-characterized examples of post-translational events that modulate protein function. However, there is also a class of larger modifications that plays an equally important role in protein regulation. This class includes tagging of proteins with ubiquitin as a signal for degradation.

In the early 1980s, Avram Hershko and Aaron Ciechanover, from the Technion-Israel Institute of Technology in Haifa, first reported that a small protein (later found to be ubiquitin) ubiquitously expressed in all cell types is a component of an ATP-dependent *in vitro* proteolytic system in rabbit reticulocyte extracts. They demonstrated that ubiquitin is covalently linked to protein substrates and that it was necessary for the degradation of these proteins in their system [1,2]. The importance of these biochemical findings was fully appreciated only a few years later, through the work of Alexander Varshavsky, Aaron Ciechanover, and Daniel Finley, then at the Massachusetts Institute of Technology in Cambridge. They used cells expressing a temperature-sensitive ubiquitin-activating enzyme to show that ubiquitin is required for protein degradation in living cells as well as for cell viability [3,4].

The ubiquitin-proteasome system is complex and consists of a highly organized cascade of enzymatic reactions that select, mark and degrade intracellular proteins. Degradation of a protein *via* the ubiquitin system involves two successive steps: conjugation and degradation [Figure 1]. The conjugation step includes a regulated and coordinated series of enzymatic reactions in which multiple ubiquitin molecules (ubiquitin is a small protein that serves as a tagging unit) are covalently linked to the target protein. In this step, E1 (ubiquitin-activating enzyme) activates ubiquitin molecule and transfers it to E2 (ubiquitin-carrier protein). In the next reaction, E2 transfers the activated ubiquitin to the target protein that is

The ubiquitin-proteasome pathway has a central role in selective degradation of intracellular proteins in all eukaryotic cells.

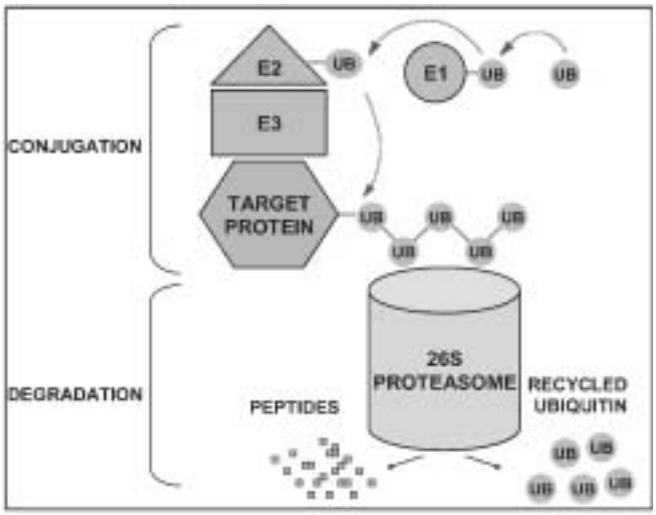

Figure 1. Degradation of proteins via the ubiquitin-proteasome system. **Conjugation:** Free ubiquitin (UB) is activated by ubiquitin-activating enzyme (E1). Subsequently, ubiquitin is transferred to one of many distinct ubiquitin-carrier proteins (E2), which transfers the activated ubiquitin to the target protein that is specifically bound to ubiquitin-ligase (E3). Multiple cycles of conjugation of new ubiquitin molecules to the previous one result in the formation of a poly-ubiquitin chain. **Degradation:** the poly-ubiquitin chain is recognized by the 26S proteasome resulting in degradation of the substrate to small peptides and recycling of ubiquitin.

specifically bound to E3 (ubiquitin-ligase). Multiple cycles of conjugation of new ubiquitin molecules to the previous one result in the formation of a poly-ubiquitin chain. In the degradation step, a large proteolytic complex – the 26S proteasome – recognizes the poly-ubiquitin chain, resulting in degradation of the substrate to small peptides and the recycling of ubiquitin. Both conjugation and degradation require metabolic energy in the form of ATP [5].

The ubiquitin-proteasome system has a hierarchical structure. Thus, the genome encodes a single E1 which activates ubiquitin molecules for all the substrates of the system. Inactivation of this gene is lethal in yeast [6]. Many species of E2 enzymes have been characterized and, typically, each E2 interacts with a number of E3 enzymes, thus becoming involved in targeting numerous substrates. The E3 enzymes are responsible for the specific recognition of the multitude substrates of the ubiquitin system. The E3 is either a protein or a protein complex that binds to both the E2 and the substrate; it interacts with the substrate directly or via ancillary proteins [5]. The 26S proteasome is a large protease complex that is composed of two sub-complexes: a 20S core particle that carries the catalytic (proteolytic) activity, and a 19S regulatory particle that recognizes the poly-ubiquitinated substrates [5,7].

Protein modification via ubiquitination is one of the most common immunity-related regulatory events, controlling various processes including transcriptional activation and antigen processing and presentation.

Degradation of proteins by the ubiquitin system plays a key role in many cellular processes, such as the regulation of cell cycle, differentiation, cellular response to stress, modulation of cell surface receptors, DNA repair, transcriptional regulation, and regulation of the immune and inflammatory response. Among the substrates of the system are cell cycle regulators, tumor suppressors, proto-oncogenes, transcription factors, and mutated and misfolded proteins [5].

With numerous cellular pathways affected, derangements in the ubiquitin system have been shown to result in various human diseases, including malignancies [8,9], neurodegenerative diseases [10], and hereditary syndromes [11,12,]. Given the fact that all mammalian cells have ubiquitin enzymes and proteasomes, selectively targeting proteins for ubiquitination and degradation, or proteasome inhibition, have potential for treatment of many human diseases. Thus far, inhibition of the proteasome has been shown to be effective in several animal models for various human diseases including cancer [13], cerebral ischemia [14,15], and inflammatory conditions such as rheumatoid arthritis [16], asthma [17], multiple sclerosis [18] and psoriasis [19]. Two proteasome inhibitors are currently under clinical evaluation. PS-341 (bortezomib) is being evaluated in multiple phase II clinical trials for several solid tumor indications and has just entered a phase III trial for multiple myeloma [20]. PS-519, representing another chemical class of inhibitors, inhibits the inflammatory events following ischemia and reperfusion injury, and is now in a phase II clinical trial for acute ischemic stroke [21].

Protein degradation drives a variety of immunity-related regulatory events, including transcriptional activation, apoptosis, and antigen processing and presentation. Some examples will be reviewed below.

The role of the ubiquitin system in antigen processing and presentation

As part of the immune surveillance system, cytotoxic T cells recognize antigens, which are bound by major histocompatibility complex class I proteins. To allow binding to MHC class I molecules, an antigen of a virally infected cell has to be proteolytically processed into peptides. The peptides with the appropriate length and a specific MHC class I binding motif bind to the transporter associated with antigen processing. They are then translocated into the endoplasmatic reticulum where they bind to the respective MHC class I molecules, and are transported to the plasma membrane. The recognition of the MHC-peptide complex on the plasma membrane by a T cell receptor eventually leads to T cell activation [22]. Although it is not the only protease involved in this process, it is widely accepted that the 26S proteasome is responsible for the generation of the majority of MHC class I peptides [23]. The strongest evidence that the proteasome generates the majority of MHC class I-presented peptides comes from studies with proteasome inhibitors. These agents have been shown to block the presentation of many different proteins and to act by specific inhibition of proteasomes and not through effects on other targets in cells. When cells are treated with proteasome inhibitors, they continue to synthesize class I molecules at normal rates; however, these polypeptides fail to assemble into stable complexes because of the lack of antigenic peptides. Therefore, proteasome inhibitors appear to block the generation of most class I-presented peptides from cell proteins [24,25].

An interesting finding is that the cytokine interferon-gamma, which stimulates antigen presentation, also leads to induction and exchange of three proteasomal subunits that lead to alteration in the cleavage site preferences of the proteasome. This "new" proteasome is referred to as the immuno-proteasome [26]. Analysis of almost 20 different MHC class I epitopes shows that the immuno-proteasome up-regulates the presentation of some epitopes – mostly viral such as the influenza A nucleoprotein [27], the hepatitis B core antigen [28], and the adenovirus E1B protein [29]. However, the generation of other viral epitopes is not affected at all. The biological significance of those findings is not completely understood.

Since the proteasome is the central source of antigenic peptides, it is not surprising that certain viruses can interfere with the proteolytic activity of the proteasome. However, since functional proteasomes are vital for cell survival, and consequently for viral propagation, viral proteins modulate proteasome activity rather

MHC = major histocompatibility complex

than completely blocking it [30]. A particularly interesting example of a viral protein that interferes with proteasomal processing is the Epstein-Barr virus nuclear antigen-1.

EBV, the first human tumor virus to be identified, is involved in the pathogenesis of Burkitt's lymphoma. EBV is known to be widespread in all human populations, with over 90% of adults being life-long carriers. Primary EBV infection is common during early childhood and is usually clinically dormant. However, when infection is delayed until adolescence or adulthood, it may cause infectious mononucleosis. Like many other viruses that are adapted to persist in the infected host, EBV replicates and hides in different cellular compartments according to its lytic or latent expression programs. The latent period is associated with the expression of nuclear antigens (EBNA1-6) and latent membrane proteins. EBV nuclear antigen-1 (EBNA1) persists for life in healthy virus carriers and is the only viral protein regularly detected in EBV-associated malignancies [31]. Unlike EBNAs 2-4, which are strong immunogens, EBNA1 is not processed and cannot elicit a T cell response. The persistence of EBNA1 contributes, most probably, to some of the pathologies caused by the virus [32]. An interesting structural feature of EBNA1 is a long repeat of the amino acids glycine and alanine. Transfer of a strong antigenic epitope from EBNA4 to EBNA1 prevented its presentation, while its insertion in an EBNA1 deletion mutant, which lacks the amino acids repeat, resulted in its presentation to the appropriate T cells [33]. Thus, the glycine-alanine repeat serves as an element that inhibits antigen processing and subsequent presentation of potential antigenic epitopes. It has been shown that EBNA1 is resistant to degradation; however, it was degraded by the ubiquitin system when the glycine-alanine repeat was deleted [34]. Thus, the evolution of this repeat enabled the virus to manipulate the ubiquitin system and evade proteolysis and subsequent presentation to the immune system.

Although there has been remarkable progress in understanding the steps involved in antigen processing and presentation, many fundamental questions remain unresolved. Understanding the mechanisms involved as well as getting a more detailed analysis of viral interference with the proteasome system may have valuable practical applications, such as blocking those viral proteins that inhibit proteasome function and optimizing epitopes for future development of vaccines.

NF-κB activation via the ubiquitin system

Nuclear factor-kappa B was first identified as a B cell-specific transcription factor involved in control of immunoglobulin kappa light chain gene expression, and has emerged as a ubiquitous, evolutionary conserved transcription factor whose activity is rapidly induced in response to pro-inflammatory stimuli. NF-κB is involved in activation of a large number of genes in response to infection and inflammation. Among the genes activated by NF-κB are cytokines, adhesion molecules, inflammatory response and stress proteins, and immune system receptors [35].

EBV = Epstein-Barr virus
EBNA = Epstein-Barr nuclear antigen
NF-κB = nuclear factor-kappa B

NF-κB is coined as a collective term for dimeric transcription factors composed of members of the Rel family of DNA-binding proteins that recognize a common sequence motif. Five mammalian Rel proteins have been identified: NF-κB1 (p105), NF-κB2 (p100), c-Rel, Rel-A (p65) and Rel-B. The most classical form of NF-κB is a heterodimer of p50 and p65. Both NF-κB1 and NF-κB2 encode precursor proteins that are much larger than the mature functional products, p50 and p52. The precursors must therefore be processed to generate the mature transcription factor [35].

NF-κB is normally sequestered in the cytoplasm of non-stimulated cells and consequently must be translocated into the nucleus to function. The subcellular location of NF-κB is controlled by a family of inhibitory proteins, IκBs (inhibitors of κB), which bind NF-κB and mask its nuclear localization signal, thereby preventing nuclear translocation [36]. NF-κB is activated by the ubiquitin system via a two-step proteolytic mechanism:

- Most studies support a model that involves post-translational processing of the precursors (p105 and p100) by a ubiquitin-proteasome-dependent reaction. Although the precise mechanism and structural motifs that dictate the processing outcome are unclear, it has been shown that a glycine-rich region (GRR) at the C-terminal domain of p50 is an important processing stop signal that probably interferes with the activity of the 26S proteasome [37,38]. As mentioned above, a related glycine-alanine repeat derived from the Epstein-Barr virus nuclear antigen-1 protein blocks the complete degradation of EBNA1 and, consequently, its presentation as an antigen to T cells. It should be noted that ubiquitin-mediated proteolysis generally yields small peptides but does not yield partial protein fragments; thus, the generation of p50 and p52 subunits from their NF-κB precursors is exceptional.

Some viruses, such as EBV, developed escape mechanisms that manipulate the ubiquitin-proteasome system in order to persist in the infected host.

- Potent activators, such as tumor necrosis factor-alpha, interleukin-1, or lipopolysaccharide, induce rapid degradation of the inhibitor IκB within minutes [Figure 2]. This degradation process consists of a series of well-characterized steps. Inducible IκB phosphorylation is one of the earliest events in the activation pathway. Phosphorylation leads to the immediate recognition of IκB by the ubiquitin system, which consequently results in the poly-ubiquitination of IκB [Figure 2]. This modification then targets IκB for rapid degradation by the 26S proteasome. The degradation of its inhibitor uncovers the nuclear localization signal of NF-κB, resulting in translocation of NF-κB to the nucleus where it initiates specific transcriptional activity [39,40]. Given the key role of NF-κB in the pathogenesis of inflammation,

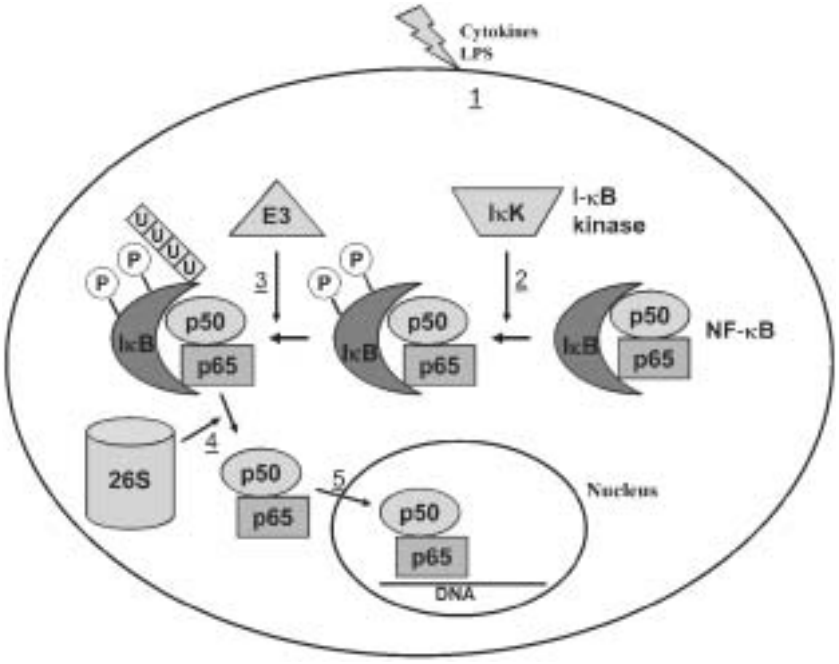

Figure 2. The NF-κB activation pathway. Cytokines such as tumor necrosis factor-alpha or bacterial lipopolysaccharide trigger the nuclear translocation of NF-κB via activation of the IκB kinase **[1]**. IκB kinase phosphorylates IκB bound to NF-κB (P), which consists of a dimer of p65 and p50 **[2]**. This phosphorylation is a signal for ubiquitination (U) of 1κB by a ubiquitin ligase (E3) **[3]** and subsequent degradation of IκB by the 26S proteasome **[4]**. The degradation of its inhibitor uncovers the nuclear localization signal of NF-κB resulting in translocation of NF-κB to the nucleus where it initiates specific transcriptional activity **[5]**.

inhibition of NF-κB activity via proteasome inhibition has shown promising efficacy in numerous animal models and in early clinical trials for inflammatory indications [21]. Future studies are required to establish whether the encouraging animal studies could be successfully applied to the treatment of human diseases.

Concluding remarks

The aim of this short review was to highlight certain aspects of protein degradation in the immune system. The presentation of antigenic peptides derived from ubiquitin-proteasome-dependent degradation of viral proteins to cytotoxic T cells is a central component of antiviral response. Therefore, it is not surprising that viruses have developed means to block proteasomal processing in order to escape detection by the host immune system. The example of Epstein-Barr virus nuclear antigen-1 was presented. Similarly, it was shown that a glycine-rich domain in p105 regulates the ubiquitin-proteasome-dependent production of the transcription factor NF-κB. These converging lines of evidence suggest that certain types of sequences in different substrates may influence their structure and capacity to associate with components of the ubiquitin-proteasome pathway. Future studies of the mechanisms underlying the production of antigens by the proteasome and the multiple strategies of viral interference with this process may lead to new therapeutic approaches for some human diseases that are linked with dysregulated proteolysis.

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