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Review

Ubiquitin-independent proteasomal degradation during oncogenic viral infections

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ABSTRACT

Most eukaryotic proteins destined for imminent destruction are first tagged with a chain of ubiquitin molecules and are subsequently dismantled by the proteasome. Ubiquitin-independent degradation of substrates by the proteasome, however, also occurs. The number of documented proteasome-dependent, ubiquitin-independent degradation events remains relatively small but continues to grow. Proteins involved in oncogenesis and tumor suppression make up the majority of the known cases for this type of protein destruction. Provocatively, viruses with confirmed or suspected oncogenic properties are also prominent participants in the pantheon of ubiquitin-independent proteasomal degradation events. In this review, we identify and describe examples of proteasome-dependent, ubiquitin-independent protein degradation that occur during tumor virus infections, speculate why this type of protein destruction may be preferred during oncogenesis, and argue that this uncommon type of protein turnover represents a prime target for antiviral and anticancer therapeutics.

Contents

1. Introduction

Protein turnover is a tightly controlled process that regulates myriad pathways to maintain cellular homeostasis and modulate the cellular environment [1–4]. The majority of protein degradation is

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accomplished by the proteasome, a large catalytic complex essential for processes such as cell cycle progression, transcription, DNA repair, apoptosis, and angiogenesis, all of which, when aberrant, can influence cellular growth control as it relates to cancer [5–12]. Furthermore, proteasome function is an essential component in the replication of many viruses, as well as in antiviral immune defenses [13–15]. Many of these viruses have been causally linked or casually associated with human cancers [13,14]. While proteasomes most often degrade substrate proteins tagged with polyubiquitin chains [16], they can also degrade non-ubiquitinated proteins [17]. Interestingly, proteasome-dependent, ubiquitin-independent degradation is prominently represented during viral infections linked to cancer, and many substrates of this reaction, in both infected and uninfected cells, are proteins that control cell growth and tumorigenesis. Below, we discuss ubiquitin-independent proteasomal degradation with a specific focus on viral infections and oncogenic transformation.

2. Protein proteasomal degradation

2.1. Proteasome components and activators

The catalytic component of the proteasome is a 700 kDa assembly that sediments in sucrose gradients with a buoyant density of 20 Svedberg units and thus is referred to as the 20S core particle (CP) [18]. It is composed of α and β subunits that form a barrel-like structure. Two sets of seven distinct α subunits form two identical rings separated by two (identical and adjacent) rings of seven distinct β subunits (Fig. 1). The catalytic surface responsible for the proteolytic processing of substrates is formed by the β1, β2 and β5 subunits and is found within the interior of the channel generated by their ring-like organization [18]. Access to these sites is restricted to proteins that can enter the narrow channel [18–21]. Proteasome activators, such as the 19S regulatory particle (RP), can associate with either or both ends of the 20S CP [22]. The combination of one 20S CP and one or two 19S RPs generates a larger complex referred to as the 26S proteasome that is responsible for the majority of protein degradation that occurs in mammalian cells [22]. The 19S RP consists of six ATPase and twelve non-ATPase subunits that mediate substrate and ubiquitin chain recognition, ATP-dependent substrate unfolding and insertion into the 20S CP, and the release of ubiquitin molecules that were covalently attached to targeted substrates [23].

In addition to the 19S RP, other proteasome activators (PA) can associate with the 20S CP to modulate proteasome function [22]. These include PA200 and three isoforms (α, β and γ) of PA28 (Fig. 2). PA28α and PA28β form a heteroheptameric complex that may play a role in the processing of antigens for display by major histocompatibility complex (MHC) class I proteins [24]. Along with interferon-inducible β subunits, it forms the immunoproteasome. PA28γ forms a homoheptamer that has been specifically implicated in the ubiquitin-independent turnover of several substrates [22]. PA200 appears to upregulate the caspase-like activity of the proteasome and may play a role in the cellular DNA damage response [25,26].

2.2. Ubiquitin-mediated proteolysis

The majority of substrates that are destined for degradation by the proteasome are covalently modified with ubiquitin molecules [16]. Ubiquitin is a 76 amino acid protein that can be covalently linked *via* its C-terminal amino acid to protein substrates at N-terminal or lysine residues. Ubiquitin chains are assembled by the addition of subsequent molecules (again *via* their C-termini) to one of seven lysine residues found within the ubiquitin molecule bound to the substrate. Often, a chain of at least four ubiquitin molecules, linked through their lysine 48 residue, promotes recognition and proteolysis by the proteasome, although there are documented exceptions to this rule [16,27]. Prior to substrate attachment or chain growth, ubiquitin monomers must first be activated by one of two E1 ubiquitin activating enzymes, subsequently transferred to one of several E2 ubiquitin conjugating enzymes, and ultimately covalently bound to target proteins with the help of one of the hundreds of E3 ubiquitin ligases. E3s provide the substrate specificity for the ubiquitination reaction [16]. In some circumstances, E4 proteins facilitate chain growth after the addition of the first ubiquitin molecule [28].

The role of polyubiquitin chains in directing covalently marked proteins for proteasomal degradation appears simply to be attaching targeted substrates to the proteasome by direct interaction with the

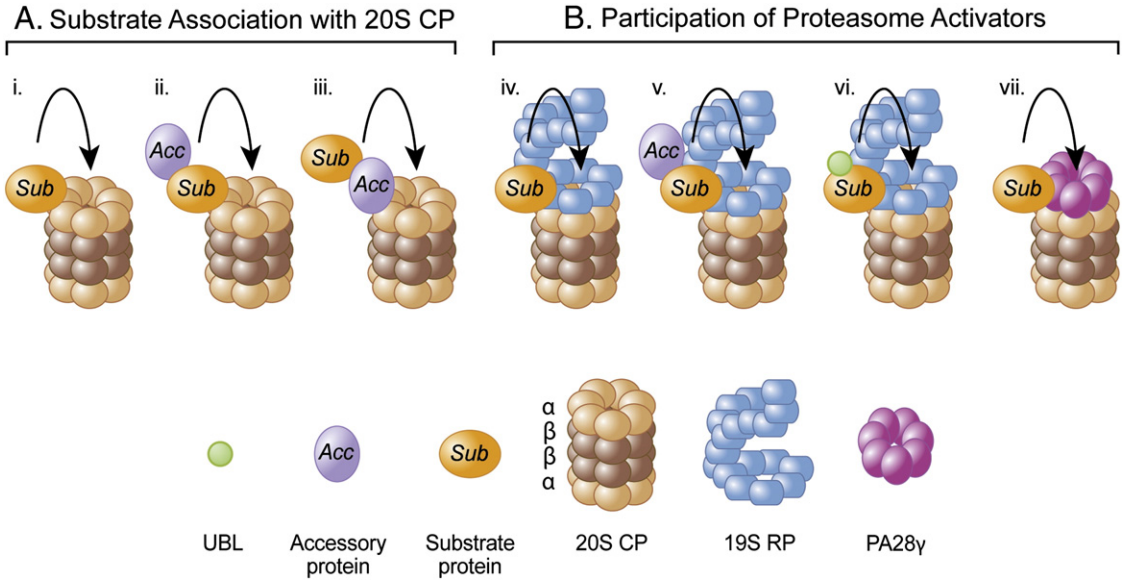

Fig. 1. Models for ubiquitin-independent proteasomal degradation. A. Substrates undergoing proteasome-dependent, ubiquitin-independent degradation often associate with the 20S CP. This association can be direct and autonomous (i), direct but mediated by an accessory protein (ii), or indirect and mediated by an accessory protein (iii). B. Other proteasome-dependent ubiquitin-independent degradation substrates associate with proteasome activators. Mechanisms include substrate association with the 19S RP either independently (iv), with an accessory protein (v), or after conjugation with a ubiquitin-like protein (vi), as well as direct association with PA28γ (vii).
Design adapted from Ref. [17].

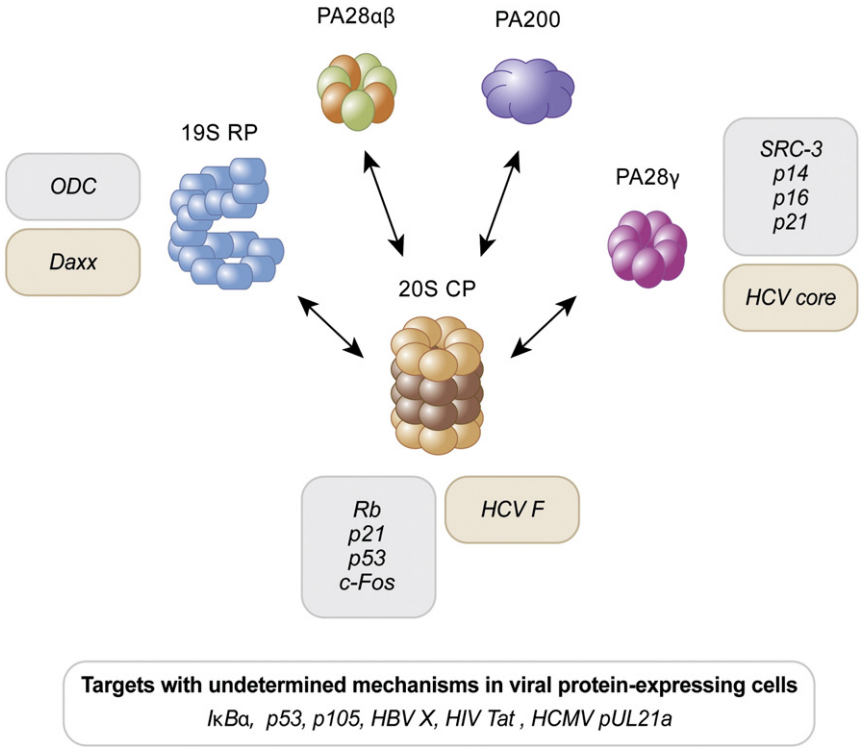

Fig. 2. Ubiquitin-independent proteasomal degradation events *in vivo*. The 20S CP alone or in association with various activators (19S RP, PA28αβ, PA200, PA28γ) mediates protein degradation (see text for details). Documented examples of ubiquitin-independent degradation events in uninfected cells (gray boxes) or in virus protein-expressing cells (yellow boxes) are displayed adjacent to the proteasomal components that mediate the event. Other proteasome-dependent, ubiquitin-independent degradation events in viral protein-expressing cells have not been ascribed to a specific proteasomal assemblage (white box).
Design adapted from Ref. [17].

Rpn13 and Rpn10 subunits of the 19S RP [29–31]. A subsequent, perhaps ubiquitin-independent step, requires partial substrate denaturation that promotes a higher affinity proteasomal association and perhaps facilitates delivery into the narrow channel leading to the protease active sites buried deep within the proteasome core [32]. Thus, ubiquitin modification acts to deliver substrates to the proteasome where modifications to their tertiary structure produce substrates amenable to efficient degradation.

2.3. Proteasomal function during oncogenesis

The proteasome mediates the majority of cellular protein turnover and is required for cellular homeostasis. Not surprisingly, deregulated protein degradation by the proteasome is observed in a variety of cancers [12]. Aberrant turnover of tumor suppressors, cell cycle regulators, as well as proapoptotic proteins, can promote tumorigenesis and/or exacerbate malignancy. Because of its important role in the induction and maintenance in tumor development, proteasome inhibition has been suggested as a broadly applicable therapeutic for cancers [12,33]. Transformed cells can be significantly more sensitive to proteasome inhibition than their untransformed counterparts. Proteasome inhibition not only induces apoptosis in transformed cell lines [34], but also sensitizes tumors to conventional therapeutics [35].

2.4. Proteasomal function during viral infection

Aberrant protein degradation is observed in a variety of cancers, including cancers induced by viral infection. Viruses are obligate intracellular parasites that commandeer proteasome function in order to degrade specific cellular targets to promote viral replication [13,14]. Collateral effects of such events sometimes perturb the cellular environment to an extent that causes disease, such as cancer. For example, the ubiquitin-dependent proteasomal degradation of p53 and the retinoblastoma (Rb) tumor suppressor by the oncogenic human papillomavirus [36] and the degradation of NF-κB by human T cell leukemia virus type 1 [37] are well-documented examples of tumor induction through viral-mediated degradation of critical cellular growth-control regulators.

Paradoxically, the proteasome is utilized both by viruses to enhance their replication [13,14,38], and by cells to generate viral peptide antigens that are presented *via* MHC molecules for immune detection and pathogen elimination [39]. Thus, while inhibiting certain proteasome-mediated proteolytic events may indeed produce antiviral effects, inhibiting others could actually exacerbate viral infections. Therefore, studying uncommon mechanisms employed by viruses to usurp proteasome function in order to promote viral replication may provide insights into ways in which these instances of proteasome function can be specifically inhibited for antiviral effect while preserving the natural antiviral effects of more native proteasomal functions.

2.5. Ubiquitin-independent proteasomal degradation

Although the majority of protein turnover mediated by the proteasome occurs through the canonical ubiquitin-dependent pathway, the number of examples of proteasomal degradation that occur without prior ubiquitination is growing [17]. Garnering acceptance that a protein is degraded in a living cell by a proteasome-dependent, ubiquitin-independent pathway is challenging because of the nature of the assays that examine this phenomenon and because many researchers are skeptical that such pathways exist or are biologically relevant. Common assays used to test for ubiquitin-independent degradation have been previously reviewed [17], and each display unique strengths and weaknesses (Table 1). Unfortunately, many of the

Table 1
Ubiquitin-independent degradation assays.

Assay	Strengths	Weaknesses
Absence of ubiquitination	Can be a visually compelling, companion experiment.	Ubiquitinated intermediates are short-lived and challenging to isolate, so negative results are difficult to interpret without extensive positive controls.
Unstable in [a]ts20 cells	Most direct and reliable assay when proper ubiquitin-dependent controls are also analyzed.	Positive controls such as p53 only show ubiquitin-dependent degradation impairment but not necessarily complete inhibition; Possibility that specific degradation events depend more highly on Ube 1L2 as opposed to the ts Ube 1 protein.
Immune to [b]DN ubiquitin	Direct and reliable assay when proper ubiquitin-dependent controls are also analyzed.	Transfection based assay in which it is difficult to achieve high-level inhibition even of positive controls.
Unstable lysine-less mutant	Simple assay for a subset of small proteins but that requires inhibitors to confirm proteasome-mediated degradation.	Larger proteins require multiple lysine mutations that may affect overall structure; Must be conscious of N-terminal-mediated ubiquitin-dependent degradation.
In vitro degradation	Defined assay favored by protein biochemists.	Inability to confirm that processes that can occur *in vitro* actually do occur *in vivo*; Technically demanding assay that requires all necessary reaction components (which may or may not be known).

[a] ts20: temperature sensitive, [b] DN: dominant negative.

assays used to examine ubiquitin-independent degradation are considered successful when a negative result is obtained [17]. For example, the absence of ubiquitin-conjugated intermediates during conditions of substrate destabilization, the failure of dominant-negative ubiquitin to inhibit substrate destruction, the inability to stabilize substrates in cells with conditional alleles of the ubiquitin activating enzyme Ube1 or when all substrate lysine residues are substituted are often used as evidence of a ubiquitin-independent pathway. While biochemical assays might show that non-ubiquitinated proteins can be degraded by the proteasome *in vitro*, they cannot demonstrate that such an event actually occurs *in vivo*. Therefore, even as examples of ubiquitin-independent degradation accumulate, the general acceptance of this pathway will only be achieved by the demonstration of detailed mechanisms that mediate these unique proteolytic events.

Ubiquitin-independent degradation generally occurs through one of two mechanisms (Fig. 1), either substrate association with the 20S CP (often with the assistance of additional proteins), or through the function of proteasome activators (Fig. 2), complexes that associate with and modulate the function of the 20S CP. Examples of 20S-mediated ubiquitin-independent degradation (Fig. 1) include structurally abnormal, misfolded, aged, or highly oxidized proteins [17]. The exposure of hydrophobic patches in such substrates likely enhances recognition by the 20S CP, and their unfolded nature likely obviates the need for 19S RP function. Such observations have led to the theory that a protein presented to the 20S CP in a partially denatured state would not require ubiquitination for degradation. *In vivo*, such proteasomal presentation may require proteins in addition to the substrate itself. For example, the HDM2 protein increases the association of two critical cell cycle regulators, Rb and p21, with the 20S CP, leading to their ubiquitin-independent degradation [40,41]. It is interesting that an E3 ubiquitin ligase, such as HDM2, normally associated with ubiquitin-dependent degradation plays such a critical role in a ubiquitin-independent pathway as well.

Ubiquitin-independent degradation has also been shown to occur with the help of known proteasome activators (Figs. 1 and 2). While the 19S RP clearly participates in ubiquitin-dependent degradation, evidence suggests that it also mediates the ubiquitin-independent degradation of ornithine decarboxylase (ODC), a critical enzyme involved in the synthesis of cellular polyamines [17,42]. In this pathway, ODC associates with antizyme, a cellular protein that inhibits its function, resulting in a conformational change that exposes a C-terminal instability element within ODC that is recognized by the 19S RP without ubiquitination, leading to the rapid turnover of ODC [43,44]. Several documented cases of ubiquitin-independent degradation are also mediated by the proteasome activator PA28γ [22], including SRC-3 [45], p21 [46], p14 [47], and the hepatitis C virus core protein [48]. PA28γ may facilitate the ubiquitin-independent degradation of these substrates by acting as a molecular chaperone that recruits substrates to the 20S CP and/or by opening the α ring in order to permit substrate insertion into the catalytic core [49].

It is interesting to note that in many of the examples cited above, the protein eliminated by ubiquitin-independent degradation plays significant roles in cell cycle progression, cellular growth control, and/or oncogenesis. Perhaps not coincidentally, accumulating evidence (Table 2) suggests that oncogenic viruses often commandeer this pathway, a process that may contribute to the oncogenic potential of these viruses. Viral oncoproteins may mediate the ubiquitin-independent degradation of target substrates in several different ways (Fig. 1). In the next sections we illuminate viral proteins that mediate ubiquitin-independent degradation, as well as viral proteins that are degraded by the cellular ubiquitin-independent pathway. Demonstrated or postulated mechanisms for these reactions (Table 2) will also be discussed.

3. Ubiquitin-independent proteasomal degradation in viral protein-expressing cells

3.1. Double stranded DNA viruses

Viruses with double stranded DNA genomes provide numerous and classic examples of oncogenic pathogens. These include the small DNA tumor viruses, such as adenovirus [50], simian virus 40 [50], and human papillomavirus [51,52], as well as herpesviruses, including Epstein–Barr virus [51], and Kaposi's sarcoma-associated herpesvirus [51]. Based on its detection in biopsied tumor samples and the pro-oncogenic functions of many of its proteins, another herpesvirus, human cytomegalovirus, has been described as oncomodulatory [53,54]. Proteasome-dependent, ubiquitin-independent protein degradation events mediated by human papillomavirus and cytomegalovirus proteins are described below.

3.1.1. Human papillomavirus

Human papillomavirus (HPV) is the etiological agent of cervical carcinoma, the second most common cancer in women worldwide, and is linked to the development of anal, perianal, vulvar, penile, and oropharyngeal cancers [52]. HPV encodes oncoproteins (E6 and E7) that bind to and induce the proteasomal degradation of key cellular tumor suppressor proteins [55]. Persistent expression of E6 and E7 allows the virus to replicate in cells that are committed to terminal differentiation, but also deregulates cell cycle checkpoints and is necessary for cellular transformation [55]. E7 degrades Rb [56], leading to the release of E2F transcription factors and subsequent

Table 2
Viruses and ubiquitin-independent protein degradation by proteasomes.

Virus class	Degradation inducer	Target substrate	Absence of ubiquitination	Unstable in ts20 cells	Immune to [c]DN ubiquitin	Unstable lysine-less mutant	*In vitro* degradation	Compelling arguments for ubiquitin-independent degradation?	Proposed mechanisms	References
[a]DS-DNA viruses	HPV E6	p53		√	√			Yes	20S [d]CP recognition of p53 upon C-terminus binding to E6	67
	HCMV pp71	Daxx		√				Yes	pp71-induced substrate binding to 19S [e]RP	98, Winkler and Kalejta (manuscript in prep)
	HCMV pp71	Rb, p107, p130	√	√	√			Yes	pp71-induced substrate binding to 19S RP	97, Winkler and Kalejta (manuscript in prep)
	?	HCMV pUL21a	√	√		√		Yes	20S CP recognition of unstructured pUL21a C-terminal domain	103
	?	MCMV M141	√	√				Yes	C-terminal [f]PEST domain as degradation signal	105
[b]SS-DNA viruses	?	MVM NS2	√	√	√			Yes	20S CP recognition of NS2 C-termius	111
Reverse transcribing viruses	?	HBV X				√		Not yet	20S CP binding; Chaperone Hdj1 participation	119–123
	$p14^{ARF}$	HIV Tat				√		Not yet	20S CP binding; PA28γ participation	133, 135–136
	HTLV Tax	IκBα				√		Not yet	Tax-mediated recruitment of IκBa to 20S CP	148–149
Positive strand RNA viruses	?	HCV core				√		Yes	20S CP binding; PA28γ participation	156
	?	HCV F		√		√	√	Yes	20S CP recognition of F (aa 20–60)	158

[a] DS-DNA: double stranded DNA [b] SS-DNA: single stranded DNA, [c] DN: dominant-negative, [d] CP: core particle, [e] RP: regulatory particle, [f] PEST: proline (P), glutamic acid (E), serine (S), and threonine (T).

expression of cellular factors that drive cell cycle progression [57,58]. E7 associates with members of a cullin-based E3 ubiquitin ligase complex [59], and it has been shown that Rb degradation upon E7 binding is ubiquitin-dependent [56].

E6 degrades p53 [60], a transcription factor that, in response to genotoxic stress, induces cell cycle arrest, apoptosis, and cellular senescence [61]. p53 is mutated in more than 50% of human cancers, attesting to its importance as a tumor suppressor [62–64]. Its levels are normally kept low in cells by the action of HDM2, a cellular E3 ubiquitin ligase [65]. Cellular stresses, such as unscheduled DNA synthesis induced by the loss of Rb proteins, activate and stabilize p53 [61]. In order to counteract effects of p53 that would be deleterious to viral replication, HPV, through its E6 protein, mediates the proteasomal degradation of p53 through multiple pathways [60,66,67]. E6 binds to E6-associated protein (E6AP), a cellular E3 ubiquitin ligase that does not target p53 in uninfected cells [66]. However, when bound by E6, E6AP promotes the polyubiquitination and proteasomal degradation of p53. E6 binds to two distinct regions within p53, a central DNA-binding domain and the C-terminus [68]. Interaction between E6 and the DNA-binding domain of p53 requires E6AP and promotes the ubiquitination and degradation of p53 [66,68].

Newer evidence indicates that p53 can also be degraded in a ubiquitin-independent manner in the presence of E6, and provides a rationale for the viral protein interacting with the C-terminus of the tumor suppressor [67]. A dominant-negative form of ubiquitin (7KR) in which all seven lysines are substituted with arginines was able to fully inhibit HDM2-mediated destruction of p53, but not E6-mediated p53 degradation. Identical results were obtained with a different type of dominant-negative ubiquitin (tUb) that represents a string of tandem, uncleavable ubiquitin molecules unable to be conjugated to substrate proteins because of an amino acid substitution (glycine to valine) at the conjugation site. Furthermore, overexpression of E6 resulted in destabilization of exogenous p53 in ts20 cells at the restrictive temperature where a temperature-sensitive E1 ubiquitin activating enzyme is nonfunctional, but was fully stabilized at the restrictive temperature when Mdm2 (the mouse homolog of Hdm2) was co-expressed. Interestingly, while neither of the dominant-negative ubiquitin molecules (7KR or tUb) was able to completely stabilize wild-type p53 in the presence of E6, both succeeded in completely stabilizing a C-terminal deletion mutant [67].

Taken together, these data strongly implicate a ubiquitin-independent mechanism of p53 degradation in the presence of E6 that co-exists with the ubiquitin-dependent degradation also induced by this viral protein. Furthermore, evidence suggests that the association of E6 with the C-terminus of p53 is critical for ubiquitin-independent degradation [67], providing a rationale for this site-specific interaction. If binding of E6 to the C-terminus of p53 were to expose an unstructured domain to the proteasome, then E6-mediated ubiquitin-independent degradation of p53 may occur through a mechanism similar to antizyme-induced ubiquitin-independent degradation of ODC. Importantly, the relevance of ubiquitin-independent, as opposed to the ubiquitin-dependent degradation of p53 during HPV infection and oncogenesis needs to be further explored.

3.1.2. Cytomegalovirus

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus that promotes significant disease in immunocompromised, immunosuppressed, and immunonaive individuals [69,70]. HCMV infection is the leading viral cause of birth defects, contributes to graft loss in transplant recipients, and leads to retinitis and blindness in acquired immunodeficiency syndrome (AIDS) patients. HCMV is also associated with certain tumors, including malignant glioma, prostate, colorectal [53], and recently breast cancer [71]. While a causative role has not been demonstrated, the presence of HCMV DNA, mRNA, and proteins in tumors, but not the surrounding healthy tissue,

Table 3
Hallmarks of cancer induced by HCMV.

Cancer hallmark	HCMV function	Examples
Sustaining proliferative signaling	[a]US28, viral infection	73–74
Evading growth suppressors	[b]pp71, [c]UL97, [d]IE1, IE2, US28	75–76
Avoiding immune destruction	UL16, UL18, UL40, UL111a, UL141, UL142, UL144, US2, US3, US6, US8, US10, US11, IE2, pp71, pp65, [e]miR-UL112-1	77–78
Enabling replicative immortality	IE1, viral infection	79
Tumor promoting inflammation	US28, viral infection	73, 80
Activating invasion and metastasis	US28, viral infection	81–82
Inducing angiogenesis	US28, IE1, viral secretome	83
Genome instability and mutation	IE1, IE2, UL76, viral infection	84–86
Resisting cell death	IE1, IE2, UL36, UL37x1, UL38, B2.7	87
Deregulating cellular energetics	Viral infection	88–89

[a] US: unique short, [b] pp: phospho protein, [c] UL: unique long, [d] IE: Immediate-early, [e] miR: microRNA.

suggests that HCMV either modulates cellular environments to perpetuate malignancy, or preferentially infects tumor cells because they may better support viral replication. HCMV infection (Table 3) induces all of the hallmarks of cancer [72–89]. Interestingly, one of the characteristics of a cancer cell, growth in the absence of mitogenic signals, is a property induced by an HCMV protein through a ubiquitin-independent, proteasome-dependent protein degradation event.

The product of the HCMV UL82 gene, a protein called pp71, is incorporated into the tegument layer of virions [90]. Thus, it is delivered to cells immediately upon viral entry where it interacts with cellular transcriptional co-repressors such as Daxx [91,92], BclAF1 [93], and the tumor suppressors of the retinoblastoma family, Rb, p107, and p130 [94], and induces their proteasomal degradation [93–95]. The degradation of Daxx [95] and BclAF1 [93] stimulates the viral immediate-early gene expression that is essential for the initiation of a productive lytic infection [70]. Not surprisingly, viruses that express pp71 mutants unable to bind [91,92] and degrade [95] Daxx have severe growth defects. pp71 also induces Rb degradation at the start of HCMV infection [96]. Interestingly, this degradation event is not required for viral replication *in vitro* [92], perhaps because HCMV also phosphorylates and inactivates Rb [96]. These multiple, possibly redundant mechanisms may make any single one dispensable [76]. Interestingly, both the degradation of Rb by HCMV pp71 and the phosphorylation of Rb by HCMV UL97 stimulate cell cycle progression [94,96]. These events may be among the many ways through which HCMV might promote oncogenesis.

When the role of ubiquitin has been examined, pp71-mediated degradation events were found to be ubiquitin-independent [97,98]. Daxx degradation by either ectopically expressed or virion tegument-delivered pp71 continued in ts20 cells at the restrictive temperature, but was inhibited by the proteasome inhibitor lactacytin under these same conditions [98]. This indicates that during HCMV infection, pp71-mediated Daxx degradation is proteasome dependent, but ubiquitin-independent. Experiments in ts20 cells with ectopically expressed proteins came to a similar conclusion for the Rb family proteins [97]. Furthermore, polyubiquitinated p130 intermediates were not detected under conditions where pp71-mediated p130 degradation was observed, and a dominant-negative ubiquitin in which lysine 48 is replaced with arginine (K48R) failed to inhibit pp71-induced p130 degradation. Notably, polyubiquitination and K48R inhibition were observed in the absence of pp71 [97], where p130 is known to be degraded by a ubiquitin-dependent mechanism [99]. The diversity of assays employed engenders confidence that pp71 mediates protein degradation without the need for prior ubiquitination.

The mechanism through which pp71 induces the ubiquitin-independent degradation of its substrates remains an enigma. Modification of substrates with ubiquitin-like molecules has been shown to target them for degradation [100]. While pp71 does induce the SUMOylation of Daxx, this ubiquitin-like modification does not appear to mediate Daxx degradation by pp71 [101]. Intriguingly, recent evidence indicates that, similar to the case of antizyme-mediated degradation of ODC [102], pp71-mediated degradation of Daxx requires the 19S RP but not other proteasome activators (Winkler and Kalejta, manuscript in preparation). Much more work will be required to clearly define a mechanism for the ubiquitin-independent degradation events catalyzed by this interesting and important viral tegument protein.

In addition to cellular substrates degraded in a proteasome-dependent, ubiquitin-independent manner during HCMV infection, the turnover of a viral protein also occurs in this way. Encoded by the viral UL21 gene, pUL21a is a lysine-less protein with a very short half-life that is stabilized in the presence of the proteasome inhibitor MG132, but not the calpain inhibitor ALLM [103]. Ubiquitinated conjugates of pUL21a have not been detected, and it is not stabilized in ts20 cells at the restrictive temperature. Thus all evidence points to ubiquitin-independent proteasomal degradation [103]. A mechanism for this degradation has not been defined, but the high proline content of the C-terminal domain may represent an unstructured region that, similar to some cellular proteins [104], may participate in its rapid degradation without ubiquitination. Interestingly, even though it is an unstable protein, it is required for efficient viral replication [103]. It will be interesting to determine how pUL21a supports HCMV replication, and if the unusual pathway to its degradation plays any role in that function.

Finally, a murine cytomegalovirus (MCMV) protein is also degraded by a proteasome-dependent, ubiquitin-independent mechanism during infection [105]. Because HCMV replication is restricted to human cells [70], MCMV is often used as an *in vivo* surrogate model [106]. The MCMV pM141 protein is rapidly turned over in the absence of pM140 [107]. It is stabilized by proteasome inhibitors but not in ts20 cells at the restrictive temperature, and ubiquitinated conjugates have not been observed [105]. Hence this appears to be another solid case of ubiquitin-independent degradation. pM141 contains a PEST sequence (a string of amino acids enriched in prolines (P), glutamates (E), serines (S) and threonines (T)) that in other proteins has been implicated in their rapid destruction [108]. Interestingly, binding to pM141 is not sufficient for stabilization [105], but an additional, highly acidic pM140 region of unknown function is also required [105].

3.2. Single stranded DNA viruses

Parvoviruses are small, non-enveloped viruses with single stranded DNA genomes that cause disease in animals by affecting the gastrointestinal tract and lymphatic system, leading to vomiting, diarrhea and immunosuppression [109]. The most identifiable member is adeno-associated virus (AAV), which is employed as a gene therapy vector and in vaccines because of its safety and the availability of simple molecular engineering techniques [110]. Although these viruses are not implicated in oncogenesis, a ubiquitin-independent, proteasome-dependent degradation event occurring in cells infected with minute virus of mice (MVM) is briefly described below.

3.2.1. Minute virus of mice

NS2, one of the two viral non-structural proteins of MVM, is a short-lived protein degraded in a proteasome-dependent, ubiquitin-independent manner [111]. It is stabilized by the proteasome inhibitors lactacystin, epoxomycin, or MG132, but not by dominant-negative ubiquitin or in ts20 cells at the restrictive temperature. In cell culture assays where ubiquitination of cyclin E was observed and where NS2 was rapidly degraded, no ubiquitinated conjugates of the viral protein were detected [111]. Thus the evidence for ubiquitin-

independent degradation is strong. While the C-terminus of NS2 is required to mediate its proteasomal degradation [111], the mechanism through which it does so is unclear. NS2-null mutant viruses show defects in capsid assembly and viral replication in certain cell types [112,113], making a further study of the ubiquitin-independent, proteasome-dependent turnover of this protein desirable.

3.3. Reverse transcribing viruses

Some viruses package RNA genomes into their capsids, but during the replication cycle convert that nucleic acid into DNA through a process called reverse transcription, and thus are referred to as reverse transcribing viruses. Examples associated with human tumors include the hepatitis B virus and retroviruses, such as the human immunodeficiency virus type-1 and human T cell leukemia virus type 1. Proteins expressed by each of these viruses induce proteasome-dependent, ubiquitin-independent degradation events that are described below.

3.3.1. Hepatitis B virus

Infection of hepatitis B virus (HBV) causes liver inflammation, jaundice, both acute and chronic hepatitis, and is strongly associated with hepatocellular carcinoma (HCC) [114]. With more than 350 million chronically infected, persistent infection with HBV is a major health problem worldwide. HBV X protein (HBX) is a multifunctional protein essential for both viral replication and the development of HCC [115,116] that acts as a transcription factor and interferes with nucleotide excision repair [116]. Transgenic mice that express HBX develop liver cancer [117]. Thus HBX appears to be a relevant target for an HBV-induced HCC therapy.

HBX is an unstable protein that is polyubiquitinated and subsequently degraded by the proteasome [118]. The responsible E3 ubiquitin ligase has not been identified. HBX is also degraded through a ubiquitin-independent process. A lysine-less mutant of HBX that is not detectably ubiquitinated is still subject to proteasomal degradation with an efficiency that mirrors the wild-type protein [119]. Interestingly, HBX interacts with the Rpt2 component of the 19S RP and multiple subunits of the 20S CP, including α4, α7 and α8 [120–122]. While this association may serve to curtail the turnover of several cellular substrates [120,121], it may also facilitate the proteasomal degradation (ubiquitin-dependent and/or ubiquitin-independent) of HBX itself. Examination of a role for proteasome binding in HBX degradation as well as the use of additional diagnostic assays would solidify the conclusion of ubiquitin-independent degradation.

In addition to proteasome binding, cellular protein chaperones may play a role in HBX degradation. Chaperones are responsible for unfolding substrates to facilitate translocation into the proteasome core particle [123], similar to a postulated role for the 19S RP [124]. Thus chaperone-induced protein unfolding may bypass the need for ubiquitination during proteasomal degradation. Overexpression of human DnaJ homologue-1 (Hdj-1)/Hsp40, a protein chaperone, induces HBX proteasomal degradation [125], as well as the degradation of other, often misfolded proteins [126]. While the ubiquitin-dependence of Hdj-1/Hsp40-induced degradation events has not been examined, it is tempting to speculate that along with direct proteasome binding, chaperone-mediated unfolding may play a part in the proteasome-dependent, ubiquitin-independent degradation of the HBX protein.

3.3.2. Human immunodeficiency virus

HIV infection often leads to the development of AIDS, a disabling, life-threatening illness. From its discovery in 1982, millions have died from AIDS, and more than 30 million people worldwide are living with HIV infection [127]. AIDS patients have a high risk of developing certain cancers, such as Kaposi's sarcoma, non-Hodgkin lymphoma, and cervical cancer [128]. The connection between HIV/AIDS and cancers is not completely understood, but the link likely depends on a weakened immune system caused by viral infection of CD4+ helper T cells [129].

Transcriptional elongation of HIV genes from the integrated provirus is dependent upon the viral Tat protein [127,130]. Tat recruits the cellular PTEF-b kinase complex to phosphorylate the C-terminus of RNA Polymerase II that stalls (at the TAR element) shortly after initiation, thus allowing it to complete transcription of the entire viral genome. As combination therapy with multiple drugs targeting different HIV proteins has proven to be very effective at delaying the onset of AIDS [131], Tat has garnered interest as a novel drug target to complement more conventional antiviral therapies, such as reverse transcriptase and protease inhibitors. Tat is polyubiquitinated on lysine 71 by HDM2, a cellular E3 ubiquitin ligase [132]. However, the ubiquitin chain is linked through lysine 63, not the lysine 48 that normally leads to proteasomal degradation [132]. Thus, ubiquitinated Tat is not destabilized, and is in fact a more potent transcriptional activator than the unmodified protein.

While HDM2 does not direct its degradation, another cellular protein, the $p14^{ARF}$ tumor suppressor, appears to destabilize Tat [133]. In ARF-null cells, Tat is stable, but ARF expression reduces the half-life of Tat in a proteasome-dependent manner. The ubiquitin-dependence of this degradation event has not been fully explored. Substitution of lysine 71 with arginine did not inhibit the ARF-mediated destabilization of a Tat-ubiquitin fusion protein [133], but this is clearly neither a direct nor sufficient test of ubiquitin-independence. ARF physically interacts with PA28γ, and is itself degraded in a ubiquitin-independent manner [47]. As mentioned above, PA28γ facilitates the proteasome-dependent, ubiquitin-independent degradation of multiple cellular and viral proteins [134]. Thus ARF might recruit Tat to the PA28γ complex to induce its ubiquitin-independent proteasomal degradation. Alternatively, direct recognition by other proteasome subunits may (also or instead) be utilized as degradation mechanism(s) because Tat interacts with α and β subunits of the 20S CP [135], as well as the TBP-1 (Tat-binding protein-1) subunit of the 19S RP [136].

3.3.3. Human T cell leukemia virus

Human T cell leukemia virus type 1 (HTLV-1) infection causes adult T cell leukemia (ATL) and the inflammatory neurological disorder HTLV-1-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) [137]. Viral infection of CD4+ T cells stimulates their clonal expansion, most likely through the action of the viral regulatory protein Tax [138]. A strong transcriptional transactivator required for efficient viral gene expression [139], Tax immortalizes human primary T lymphocytes [140,141] and can cooperate with Ras to transform primary rat fibroblasts [142]. The oncogenic potential of Tax is largely attributed to its ability to constitutively activate NF-κB, an event that may be critical for the transformation and survival of cells infected with HTLV-1 [37,143].

Transcription factors within the NF-κB family regulate cell growth, differentiation, and survival by binding to the promoters of specific cellular genes and activating their transcription [144]. They themselves are held in check by the IκB family of inhibitory proteins that interact with NF-κB members in the cytoplasm and prohibit their translocation to the nucleus [145]. Latent NF-κB bound to IκB can be activated in at least two ways, the canonical pathway (where IκB is phosphorylated, then polyubiquitinated, and subsequently degraded by the 26S proteasome) and the non-canonical pathway (where specific IκB family members are phosphorylated, then polyubiquitinated and subsequently proteolytically processed by the proteasome into active transcription factors).

It is well established that Tax activates NF-κB through the canonical pathway by inducing the phosphorylation, ubiquitination, and degradation of IκBα [143,146,147]. Mutations at phospho-acceptor residues required for targeting IκBα to the proteasome are resistant to Tax-mediated degradation in transient transfection assays [146], and mutants with arginine substitutions at the lysine residues that when

ubiquitinated lead to IκBα degradation, act as dominant-negative inhibitors that prevent Tax-mediated induction of NF-κB activity [147].

Recent evidence implicates Tax in ubiquitin-independent proteolytic events involving the proteasome. Tax was found to act as a molecular bridge between IκBα and the β4 subunit of the 20S CP [148]. Furthermore, assays with potentially higher sensitivity than those previously conducted [146,147] detected Tax-mediated degradation of phosphorylation- and ubiquitination-resistant mutants of IκBα [148]. However, proteasome subunit association and the degradation of these resistant IκBα mutants have yet to be functionally linked. Tax has also been observed to associate with the 20S CP α3 subunit and the p105 subunit of IκB, leading to p105 proteasomal processing [149]. Once again, a definitive link between proteasome subunit binding and IκB processing has not been established. However, it seems possible that Tax may invoke ubiquitin-independent mechanisms to target IκB to proteasomes for activation of both the canonical and non-canonical NF-κB pathways. Finally, Tax is also implicated in the degradation of Rb through promoting its association with the 20S CP [150]. Clearly more experimentation is required to specifically demonstrate the ubiquitin-independence of these interesting events, and to elaborate their detailed molecular mechanisms.

3.4. Positive strand RNA viruses

Viruses with single stranded RNA genomes of the same polarity that are translated into proteins are called positive strand RNA viruses. Examples include the rhinoviruses that in part are responsible for common colds [151], polioviruses that cause paralysis but are nearing eradication due to vaccination efforts [152], and the hepatitis C virus that, similar to HBV (see above), causes liver disease and liver cancer [153]. At least two HCV proteins are targeted for proteasome-dependent, ubiquitin-independent degradation through processes described below.

3.4.1. Hepatitis C virus

Worldwide, over 300 million people are infected with hepatitis C virus (HCV), which can lead to hepatic steatosis, cirrhosis, and HCC. The genome of HCV encodes a single polyprotein that is cleaved by host and viral proteases to generate approximately ten distinct viral proteins [153]. One of these, the HCV core protein, modulates cellular processes such as transcription, cell cycle progression, apoptosis, and immune responses, and is implicated in viral pathogenesis, including the development of HCC [154].

HCV core protein is degraded by the proteasome after polyubiquitination at N-terminal lysine residues by E6AP [155]. Because knock-down of E6AP increases virus production, the E6AP-mediated degradation of the core protein appears to be a cellular antiviral defense mechanism [155]. Similar to other proteins discussed above, HCV core also appears to undergo ubiquitin-independent degradation *via* the proteasome. A lysine-less mutant is not detectably ubiquitinated yet is still relatively unstable and its half-life increased upon proteasome inhibition [156]. HCV core associates with the PA28γ [48,157], and expression of PA28γ accelerated the turnover of both the wild-type and lysine-less mutant proteins that were stabilized in the presence of the proteasome inhibitor MG132 [156]. Importantly, E6AP expression enhanced the degradation of wild-type core, but not the lysine-less mutant. Conversely, knockdown of PA28γ but not E6AP stabilized the lysine-less mutant, while knockdown of either stabilized wild-type core [156]. These results strongly implicate PA28γ in the ubiquitin-independent proteasomal degradation of the HCV core protein. The significance of this event for viral infection or HCV pathogenesis remains to be explored.

The HCV F protein also appears to be degraded in a proteasome-dependent, ubiquitin-independent manner [158]. HCV F is a short-lived protein of unknown function that interacts in various assays with the α3 subunit of the 20S CP [158]. This particular subunit has a flexible N-terminal extension that regulates substrate access to the central catalytic chamber of the 20S CP [159]. Sequences upstream (aa 20–40) and including the α3 binding domain from the F protein (aa 40–60) are sufficient to cause protein destabilization when fused to GFP [158]. This implies that the degradation of this fusion protein (and by extension, wild-type F) may be facilitated simply by the physical interaction between F protein and the α3 subunit of the 20S CP without the need for polyubiquitination. Perhaps this association induces a conformational change in α3 that permits the delivery of F to the proteolytic active sites buried deep within the proteasome core. Indeed, the domain fused to GFP in these experiments lacked lysine residues, and a lysine-less derivative of F is no more stable than the wild-type protein [158]. HCV F was also not stabilized in ts20 cells cultured at the restrictive temperature, but was degraded by purified 20S CP *in vitro* in the absence of ubiquitination machinery. Thus it appears likely that HCV F is degraded in a proteasome-dependent, ubiquitin-independent manner. Similar to the viral core protein, the role that this might play during viral infection of HCC development has not been explored.

4. Conclusions

Most proteins degraded by proteasome-dependent, ubiquitin-independent pathways are involved in growth control processes that, when disrupted, can promote oncogenesis. This is true for both uninfected and virus-infected cells and tumors. One questions whether this seeming over-representation is pathologically relevant or simply a result of the prevalence of cancer research programs and the intense focus on understanding tumor cell biology and the roles of viruses in cancer etiologies.

That caveat aside, here we speculate on why ubiquitin-independent degradation appears to be a prominent component of cancer cell biology. A first point to ponder is why select tumor suppressor proteins are degraded by both ubiquitin-dependent and ubiquitin-independent pathways. Ubiquitin-independent degradation does not require the enzymatic cascade of ubiquitin-conjugation and thus may be faster and more efficient than ubiquitin-dependent degradation. Accelerated and economical protein degradation may be important during embryonic or stem cell cycles and become cryptically activated in virus-infected or tumor cells. Independent mechanisms may also provide a back-up or fail-safe plan to increase the probability that required proliferative events would be realized.

One must next consider why these ubiquitin-independent events are observed more prominently in cancer cells than in normal cell cycles. With fewer mechanistic steps and an apparent lesser potential for regulation, this pathway for protein destruction may be more susceptible to activation through mutation than the multi-component, tightly regulated ubiquitin-dependent process. In addition, cancer cell energetics may favor a more energy-efficient method for at least a subset of the protein destruction that drives cell division. A related point is the significant association of ubiquitin-independent degradation events associated with viral infections. As virus-infected cells and transformed cells can be energetically and metabolically similar, perhaps the same characteristics that may promote association with cancer (ease with which regulatory mechanisms can be bypassed; resource conserving (*e.g.* ubiquitin pools)) also make ubiquitin-independent degradation a palatable alternative for viruses. A further consideration is that facilitating the ubiquitin-independent degradation of their own protein products may allow viruses to evade adaptive immune responses by keeping protein steady-state concentrations low, especially if the peptides produced by this mode of proteasomal degradation were less well incorporated into MHC molecules for extracellular presentation, thus disguising the infected cell from immune recognition. There is precedence for this type of viral strategy to avoid immune detection, as sequences in the EBV protein EBNA1 impair its proteasomal degradation and thus its presentation by MHC molecules [160].

A final question is whether or not proteasome-dependent, ubiquitin-independent degradation is a suitable target for anti-cancer and/or anti-viral therapeutics. Proteasomal degradation itself is clearly a valid target, as the proteasome inhibitor bortezomib is used clinically for the treatment of mantle cell lymphoma and multiple myeloma [33]. Therefore, one might suspect that specifically inhibiting the ubiquitin-independent proteasomal degradation events that appear to preferentially target cellular tumor suppressors and viral oncogenes could approach the therapeutic effect of complete proteasome inhibition without invoking as many deleterious side effects. As many viruses are inhibited *in vitro* by proteasome inhibitors [161–165], curtailing ubiquitin-independent degradation events certainly has therapeutic potential for viral infections. Because PA28γ plays a conspicuous role in many ubiquitin-independent degradation events, modalities that inhibit its expression, function, or prevent it from associating with the 20S CP could provide therapeutically relevant inhibition.

In summary, the documentation and acceptance of proteasome-dependent, ubiquitin-independent degradation events are ever increasing. This relatively uncommon method of protein destruction is inextricably tied to viral infections and cancers. The description of more detailed mechanisms for a larger number of these events will advance their more general recognition and appreciation, as well as unveil targets for interventions to potentially treat infectious and proliferative diseases.

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