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Ubiquitin-Proteasome System as a Modulator of Cell Fate

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Summary

The ubiquitin/ proteasome system is the major non-lysosymal system for degrading proteins in the cell; the work leading to its discovery was awarded the Nobel Prize in Chemistry in 2004. In addition to small ubiquitin-like modifiers (e.g. Sumo and Nedd8), ubiquitin is involved in the complex regulation of the levels and function of many proteins and signaling pathways involved in determining cell fate. The cell death regulatory proteins, such as Bcl-2 family proteins and caspases are targeted for degradation by the ubiquitin proteasome system (UPS). In addition to mediating the degradation of proteins, the UPS regulates function and translocation of proteins, many of which play a role in the determination of cell fate. For example the UPS can regulate the activity of transcription factors, such as P53, NF-κB and HIF-1α, which control the expression of protein mediators of cell death. Aberrant UPS function has been reported in multiple neuropathologies including Parkinson's diseases and ischemia. With the number of ubiquitin conjugating and de-conjugating enzymes reaching close to the levels of protein kinases and phosphatases, it is clear that ubiquitination is an important biological regulatory step for proteins.

Introduction

Protein degradation by ubiquitin-proteasome system (UPS) is strictly-ATP dependent, and in most cases requires the presence of an ubiquitin chain on the substrate protein [1]. The ubiquitination and proteasomal degradation of proteins enables the rapid regulation of protein levels [2-4]. In addition, mono- and poly-ubiquitination of proteins can result in their translocation, internalization and modification of function.

Protein mediators of apoptosis are regulated by the UPS, via direct or indirect modulation of proteins associated with cell death (for an additional review see [5]). Here we will review recent findings on the regulation of cell death mediating proteins and signaling pathways by the UPS. In addition, we discuss the role of the UPS in the cellular response to two neuropathologies, Parkinson's disease and ischemia (stroke).

The ubiquitin-proteasome system regulates levels of the *Bcl-2* family of cell death regulatory proteins

The Bcl-2 super family regulates the balance of cell fate, via their opposing actions at the mitochondria. Multiple pro-survival Bcl-2 family members are degraded by the UPS system; the best examples are the pro-survival members Bcl-2 and Mcl-1. Bcl-2 is degraded by the

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UPS, although the role of phosphorylation is not yet clear. Basu and Haldar reported that phosphorylation of Bcl-2 on Ser70, Ser87 result in its ubiquitination and degradation [6]. Interestingly, mutation of these residues preventing Bcl-2 phosphorylation also prevents the interaction of Bcl-2 with Pin1-α (cis-trans peptidyl prolyl isomerase). In contrast, phosphorylation of Bcl-2 by mitogen activated protein kinase/ extracellular regulated protein kinase (MAPK/ ERK) on Thr56, Thr74 and Ser87, prevents its degradation [6,7]. Nitric oxide (NO) regulates sensitivity to cisplatin (anti-tumor drug) via the s-nitrosylation of Bcl-2. S-nitrosylation of Bcl-2 prevents its ubiquitination and subsequent degradation [8]. NO donors increase Bcl-2 levels and NO inhibitors decrease Bcl-2 levels [9]. Hence these mechanisms may provide therapeutic targets for promoting or reducing Bcl-2 function, but also provide an additional biological effect of NO system modulators.

Bax, Bid and Bim protein levels are all regulated by the UPS, although the most studied to date is Bim. Phosphorylation of Bim on Ser87 by Akt prevents its degradation, via its association with the chaperone protein 14-3-3 [10]. Phosphorylation of Bim on Ser65 by MAPK/ERK promotes its ubiquitination and degradation [11,12]. However, phosphorylation of Bim by JNK on the same residue (Ser 65) activates Bim promoting cell death [13]. Bim contains a MAPK/ ERK docking domain (DEF domain), which may account for its interaction with MAPK/ ERK [14]. Consistent with the findings of Ley et al, [12] brief ischemia results in Bim degradation by the UPS mediated by MAPK/ERK, which may be part of an endogenous protective mechanism of neurons to ischemia (rapid ischemic tolerance) [4].

What is unclear from the studies of pro- and anti-apoptotic Bcl-2 proteins is the nature of the E3-ligases that regulate their ubiquitination. The ubiquitination of Bax and Bid do not rely on their interaction with other Bcl-2 proteins [15], which suggests additional proteins are the E3-ligases. In addition, the conformational shift in Bax following apoptosis induction appears to prevent its ubiquitination [5]. Mcl-1, but not Bcl-2, binds to and is ubiquitinated by a BH3 domain containing E3-ligase (ARF-BP1). Furthermore, the interaction between Mcl-1 and ARF-BP1 can be blocked using a competitive exogenous BH3 domain containing protein [16]. This suggests that different E3-ligases exist for different Bcl-2 family members. The specific regulation of individual Bcl-2 family members via their E3-ligases has implications for disease therapeutics based on promoting or retarding cell death, such as cancer and stroke.

Regulation of the extrinsic cell death pathway by the UPS

The extrinsic cell death pathway mediates the toxic signaling of TNFα and other death receptors, and has multiple sites of regulation by the UPS. Activation of death receptors leads to the formation of a multi-protein intracellular signaling complex (disc-death inducing signaling complex), which promotes the activation of caspase 8 and initiation of cell death cascades. Ubiquitination of receptor interacting protein 1 (RIP1) a death receptor interacting protein, inhibits TNF-induced cell death [17]. In the absence of ubiquitination, RIP1 serves as a pro-apoptotic-signaling molecule by engaging caspase 8. Therefore, RIP1 is a dual-function molecule that can be either pro-survival or pro-death depending on its ubiquitination state.

The role of cellular FLICE-inhibitory proteins (cFLIP) in apoptotic signaling has been controversial [18]. Data obtained from cells stably over-expressing cFLIP and from mice deficient in cFLIP, support an anti-apoptotic role. cFLIP exists as multiple alternative spliced isoforms: $cFLIP_S$, consisting only of two death effector domains and $cFLIP_L$, a caspase-8 homologue, which lacks specific amino acids for proteolytic caspase activity. cFLIP is induced by NFκB in response to pro-survival TNFα signaling. cFLIP blocks toxic TNFα and Fas-mediated signaling in tumors by blocking caspase 8 activation. However, toxic TNFα-mediated JNK activation promotes cFLIP ubiquitination by the E3-ligase itch and its subsequent proteasomeal degradation [19]. Levels of cFLIP are reduced by the cyclin dependent kinase

inhibitor flavopiridol [20] or via an indirect effect of proteasome inhibitors inducing cell cycle arrest [5]. cFLIP can also regulate the ubiquintation of other signaling pathways. cFLIP blocks the ubiquitination of β-cateinin and Hypoxia Inducible Factor −1α (HIF-1α) promoting Wnt system signaling and hypoxia-mediated gene expression [21].

In addition to regulating upstream components of cell death signaling, the executioner caspases 3 and 7 can bind and are ubiquitnated by members of the E3-ligase containing inhibitor of apoptosis family [5]. Therefore the ability of a cell to activate cell death signaling cascades can be regulated at multiple sites following the degradation of key regulatory proteins by the UPS.

Regulation of P53 by the UPS

Perhaps no protein shows the many complex mechanisms to regulate protein function better than the tumor suppressor P53. P53 is regulated by phosphorylation, sumoylation, acetylation, methylation and ubiquitination. P53 is activated by a variety of stressors, which stabilize the protein. Depending on the context of the stimuli, activation of P53 can result in cell cycle control, senescence and apoptosis. Here we will focus on its ubiquitination/ sumoylation and the result on apoptosis (For a comprehensive review of P53 regulation [22]).

P53 is ubiquitinated by mouse double mutant 2 (MDM2) or in humans Human double mutant 2 (HDM2), which is self-regulated by auto-ubiquitination. However, recent studies show that additional proteins regulate the ubiquintation of P53, including MdmX, HAUSP, ARF, COP1, Pirh2, and ARF-BP1 (reviewed in [23]). P53 can be activated by ARF following the ubiquitination and/ or sumoylation of MDM2 adding yet more complexity to the regulation of the system. In addition, ARF inhibits the activity of ARF-BP1, -to increase P53 stability. Many of the proteins/ E3-ligases which regulate P53 have E3-ligase functions for multiple substrates, e.g. ARF-BP1 was identified as the E3-ligase for the pro-survival gene Mcl-1, and MDM2 has been shown to ubiquitinate the synaptic protein PSD-95 [16,24]. The apparent redundancy of the system is not yet clear and currently subject to further clarification.

P53 can be ubiquitinated on at least 6 lysine residues. The E3-ligase responsible for mono-ubiquitination of P53 is not clear. MDM2 may function as the E3-ligase adding a single ubiquitin and additional proteins may function as the E4-ligase, for example CBP/P300, although this is not supported by all data. Alternatively, low levels of MDM2 vs. P53 may mediate mono-ubiquitination, where as higher levels of MDM2 may mediate poly-ubiquitination [25]. This would make mono-ubiquitinated P53 a key regulator of cell survival/ cell death.

Activation of P53 increases the expression of the cell death genes Bax, Noxa and Puma that may lead to apoptosis. However, mono-ubiquitination regulates protein translocation, including P53 mitochondrial translocation for a transcription-independent death program [26] and the nuclear translocation of PTEN and FOXO4. Interestingly, mono-ubiquitination of P53 can enhance its sumoylation, promoting nuclear export. The direct mitochondrial death promoting effect of P53 occurs quicker than induction of Puma gene expression. P53 may block the effect of pro-survival Bcl-2 members such as Bcl-2/ Bcl-XL via their binding to the P53 DNA binding domain, which tends to be mutated in tumors. P53 forms a complex with the pro-cell death protein Bak, by displacing Mcl-1. When P53 reaches the mitochondria it can be de-ubiquitinated by HAUSP, generating non-ubiquitinated P53, which is an important step to interact with Bcl-2/ Bcl-XL. This data suggests the mitochondrial function of P53 may be part of an initial response to a harmful stress, which is supported later by nuclear actions of P53.

The role of the UPS in Parkinson's disease

Interest in the role of the UPS in Parkinson's disease is generated from the observation that out of 10 disease related genes one is an E3-ligase, parkin (PARK2), and one a deubiquitinating enzyme (PARK5) [27]. Mutations in these genes may lead to a susceptibility to UPS failure resulting in protein accumulation Lewy body formation and dopaminergic cell death. Interestingly, the dopaminergic neuron-toxin 6-OH dopamine increases protein ubiquitination and Lewy body formation. Proteasome malfunction is reported in Parkinson's disease [27], and proteasome inhibitors have been used to model Parkinson's disease in rats (although see [28]).

The E3-ligase Parkin protects cells from ER and oxidative stress, and is upregulated in response to tunicamycin and 2-mercapto ethanol induced ER stress in certain cell types [29]. Parkin has recently been shown to interact with Lim kinase 1, in addition to HSP70 and CHIP[30]. Parkin mutants are associated with a defective cytoskeleton and impairment of vesicular protein transport. The activity of Parkin is enhanced by the E3-ligase/ chaperone protein CHIP [30]. Parkin catalyzes mono-ubiqutination of protein substrates, whereas the ubiquitin-protein ligases CHIP and MDM2 promoted the formation of poly-ubiquitin chains [31]. CHIP functions as a U-box-dependent E3-ligase and promotes the degradation of other chaperone proteins such as HSC70 [32]. The chaperone function of CHIP is temperature dependent, and increases following heat stress [33]. In this manner CHIP may perform a triage role for unfolded or misfolded proteins. Indeed CHIP and HSC70 ubiquitinates phosphorylated tau, which accumulates in Lewy bodies in Parkinson's disease. CHIP also regulates the degradation of ASK-1 (apoptosis signal-regulating kinase 1) a mitogen-activated protein kinase (MAPK) that transduces apoptotic signals [34]. The degradation of ASK-1 by CHIP is blocked by Gα13 [34]. Parkin may also be a novel activator of IKK/NF-κB signaling, suggesting that Parkin mediates a neuroprotective effect by modulating ubiquitination of the IKK/NF-κB pathway [35]. Hence CHIP may play a protective role to prevent aberrant protein folding and reduce cell stress.

Parkin may, however, play a more active role in neurons via its interactions with ion channels. Parkin interacts with the NMDA receptor signaling complex, via a C terminus PDZ domain, which is lost in the mutated form associated with Parkinson's disease [36]. Acid sensing ion channel (ASIC) activity is regulated by the Parkin-mediated mono-ubiquitination of PICK1 (protein interacting with C-kinase 1)[37]. Both NMDA receptors and ASIC channels are associated with cell death in ischemia [38]. The interaction between ASICs and Parkin is also via the PDZ domain. This suggests that Parkinson's disease associated mutations of the Parkin PDZ domain may result in aberrant ASIC and NMDA signaling, therefore promoting excitotoxicity. Clearly further work will identify additional Parkin-interacting proteins, and additional pathways under its regulation.

Ischemia, the response to hypoxia and the UPS

Following harmful ischemia there is an accumulation of ubiquitinated proteins in damaged brain regions. The accumulation of ubiquitinated proteins is reduced if the brain is preconditioned with non-toxic ischemia [39] suggesting that following harmful ischemia there is a blockade or overwhelming of the UPS. Indeed it was recently reported that expression of an ubiquitin mutant UBB (+1) increases in CA1 neurons following harmful global ischemia, inducing accumulation UBB (+1)-modified proteins. However, in contrast proteasome inhibitors block ischemia induced cell death [40], although part of this effect is attributed to inhibition of toxic NF-κB signaling and inflammation. Brief non-toxic doses of ischemia promote the degradation of the pro-cell death protein Bim by the UPS, in a rapid ischemic tolerance paradigm [4]. Hence under conditions of mild and reversible ischemia, the UPS may

have a protective role, but following toxic levels of ischemia, the UPS is overwhelmed and functions to promote cell death.

HIF regulates gene expression and a wide variety of cellular responses to hypoxia. The 3 forms of HIF (HIF1, 2 & 3) are α/β heterodimers. HIF-1α is rapidly degraded in the presence of abundant oxygen due to a conserved Oxygen Dependant Degradation Domain (ODDD) with sites for trans-prolyl-hydroxylation, a modification essential for degradation of the α-subunit by the UPS [41]. Following hypoxia, HIF-1α prolyl-hydroxylation decreases, reducing HIF-1α ubiquitination and enabling its nuclear translocation, where it interacts with HIF-1β to form an active transcription factor. Following hypoxia HIF-1α mRNA and protein levels rise significantly. The von-Hippel Lindau tumor factor (VHL) facilitates the interaction of the E3-ligase with prolyl-hydroxylated HIF-1α. Loss of VHL function (by mutation or epigenetic silencing) leads to HIF-1α accumulation and reprogramming of glucose and oxygen metabolism [42], which may account for the shift from aerobic to anerobic metabolism in tumor cells, the so-called Warburg effect.

In response to hypoxia, HIF-1α is sumoylated [43]. The consequence of this, specifically in regards to transcriptional activity and stability, is controversial [44,45]. However, HIF-1α is not the only sumoylated protein in response to low oxygen conditions. During mammalian hibernation, where the brain is subject to ischemic conditions no CNS damage occurs. Extensive sumoylation of high molecular weight proteins occurred in almost all tissues, including the brain during torpor [46]. This effect was considerably more pronounced for Sumo-1 than Sumo-2/3. In addition, overexpression of the Sumo-conjugating enzyme Ubc9 increased the levels of Sumo-1 conjugates and cell survival in SHSY5Y cells following ischemia in vitro [46]. It was subsequently shown that transient global ischemia in mice, and incubation of HT22 cells with arsenite, increases the levels of high molecular weight Sumo-2/3 conjugates [47]. What remains unclear is whether sumoylation of proteins following ischemia is part of a protective process or the cause of cellular stress promoting cell death.

Conclusions

Through the regulation of protein turnover, the proteasome is involved in the control of gene expression, cell cycle regulation, apoptosis and inflammatory processes. Because of this large plethora of functions, proteasome inhibitors are the focus for future drug applications and thus clinical use, specifically in therapies for cancer and stroke [48,49]. However, global blockade of proteasome function will result in unwanted clinical side effects, hence alternative techniques to regulate proteasome function must be sought. The identification of specific E3-ligases will enable the more selective targeting of a single or small number of ubiquitin substrates as therapeutic targets. Alternatively, the targeted use of small molecules to specifically induce ubiquitination and proteasomal degradation of a target protein, or protection of a target from the UPS has recently been described [50]. These novel approaches will enable the development of a second generation of therapeutic tools and targets based on the UPS.

The ubiquitin-proteasome system and ubiquitin-like modifiers

Ubiquitin is an 8-9 kDa protein present in all cells and is attached to proteins by the sequential action of E1-, E2- and E3-ligase proteins [51,52]. E3-ligases contain either a HECT (homologous to E6-AP carboxy terminus) or RING (really interesting new gene) finger domain, a common feature of many different proteins, which catalyses the transfer of the ubiquitin from the E2 to a Lys residue on the substrate protein [51]. Small RING domain containing proteins form multimeric complexes, such as the Skp1-Cullin-F-box protein, whereas larger RING domain containing proteins can function as a single subunit [51]. The specificity of the ubiquitination reaction is regulated by the E3-ligases [51,53].

Recently identified E4-ligases may promote ubiquitin chain formation, poly-ubiquitination [54].

The pattern of ubiquitin conjugation to its target protein results in different consequences for the cell. K48 poly-ubiquitination (involvement of the K48 ubiquitin lysine residue) targets the protein to the proteasome, via a shuttle protein P62/sequestome. K29 poly-ubiquitination results in protein degradation via the proteasome, but K63 poly-ubiquitination does not and may involve the lysosome. Mono-ubiquitination of proteins can change protein complex formation, for example mono-ubiquitination of PSD-95 results in disassociation of PSD-95 from the post synaptic density [24], or results in the translocation of proteins between cell compartments, for example mono-ubiquitinated transcription factor P53 translocates to the nucleus [26]. Multiple mono-ubiquitination results in endocytosis of receptor tyrosine kinases and their lysosymal degradation [55]. With such complexity, it appears that ubiquitination of a protein can result in more subtle changes in function and stability than other posttranslational modifications, such as protein phosphorylation.

The proteasome

Proteasomes consist of a large proteinase complex with multiple catalytic domains that are located in the cytosol as well as the eukaryotic cell nucleus [56]. The S26 proteasome is a multi-catalytic protease complex consisting of an S20 core, and S19 regulatory particle, or cap. The 20S catalytic subunit contains six unique ATP-dependent serine protease sites, divided equally between chymotrypsin, trypsin and caspase-like activities. The biological importance of the proteasome is defined by the observation that deletion of 13 out of 14 genes which make up the core S20 complex result in lethal phenotypes [53,57].

Ubiquitin-like modifiers (ULMs)

In addition to ubiquitin, there is a small family of ubiquitin-like modifiers. Small ubiquitin-related modifier (Sumo-1) shares only 18% sequence identity with ubiquitin [58], where as Nedd8 (neural precursor cell expressed developmentally downregulated-8) shares an 80% sequence homology with ubiquitin [59]. It is not the sequence per se which makes these proteins function in a similar manner to ubiquitin, rather their apparent 3-D topologies. Both Sumo and Nedd8 have nearly superimposable 3-D structures compared to ubiquitin. Both proteins covalently attach to larger proteins, via a GlyGly motif at their c-termini and bind to the ε-amino group of lysine residues on acceptor proteins [58]. Nedd-8 and Sumo-2 result in single addition of a molecule to the target, where as Sumo 2/3 can result in poly-chains being conjugated to the substrate. ULM proteins also have a similar complex complement of E1 and E2 proteins that mediate their conjugation to target proteins. For a more detailed review of ULMs see [59].

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