Chapter

Ubiquitin-Independent Proteasomal Degradation Mediated by Antizyme

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Abstract

Most of the proteins in eukaryotic cells are degraded by the proteasome in an ubiquitin-dependent manner. However, ubiquitin-independent protein degradation pathway by the 26S proteasome exists in the cells. Ornithine decarboxylase (ODC) is a well-known protein that is degraded by the 26S proteasome without ubiquitination. Degradation of ODC requires the protein, "antizyme (AZ)," that is induced by polyamine and binds to the ODC monomer to inhibit ODC activity and target it to the 26S proteasome for proteolytic degradation. Namely, AZ contributes the feedback regulation of intracellular polyamine level. ODC has been considered to be the only protein that AZ binds and accelerates its degradation. However, recently AZ-mediated proteasomal protein degradation will gradually increase. Most recently, we found that one of the antizyme families, AZ2, accelerates c-Myc degradation by the proteasome without ubiquitination. In this chapter, we introduce latest several ubiquitin-independent proteasomal degradation mediated by antizyme.

Keywords: antizyme, ubiquitin-independent degradation, ornithine decarboxylase, 26S proteasome, polyamines, c-Myc

1. Introduction

In eukaryotic cells, intracellular protein degradation is mainly regulated by the ubiquitin-proteasome system, where abnormal and unwanted proteins are targeted by polyubiquitin, which is produced from monoubiquitin by ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzyme (E2) [1]. The proteins that conjugated polyubiquitin by ubiquitin ligase (E3) are finally targeted to the 26S proteasome [2]. However, there is accumulating evidence that ubiquitin-independent proteasomal protein degradation pathway exists in the cells [3, 4]. Although ubiquitin-dependent proteasomal protein degradation is carried out normally by 26S proteasome, there are many reports that ubiquitin-independent proteasomal protein degradations are executed by the only 20S proteasome without the energy of ATP hydrolysis [4]. Among others, some ubiquitin-independent degradation pathways are known to be carried out using not the 20S but the 26S proteasome with the energy of ATP hydrolysis. In this chapter, we introduce ubiquitin-independent proteasomal degradation pathway mediated by polyamine regulating protein, "antizyme."

2. What is antizyme?

Polyamines are highly charged bioactive substances presented ubiquitously in species from bacteria to human. Polyamines are necessary for cell growth and are involved in highly diversified cellular functions such as cell division, apoptosis, autophagy, oxidative stress, and ion channel activity. There are three major polyamines, putrescine, spermidine, and spermine, in the cells [5, 6]. Intracellular polyamine concentration is highly regulated by the protein "antizyme" [7–10] that is widely distributed from yeast to human [11]. Antizyme (AZ) is induced in response to the increased concentration of intracellular polyamines through the polyamine-induced translational frameshifting mechanism [12]. AZ mRNA consists of two ORFs (ORF1 and ORF2). In the low polyamine concentration, translation of ORF1 is terminated at stop codon "UGA" of ORF1, and short product is produced (**Figure 1**). But in the increasing cellular polyamine concentration, reading frame

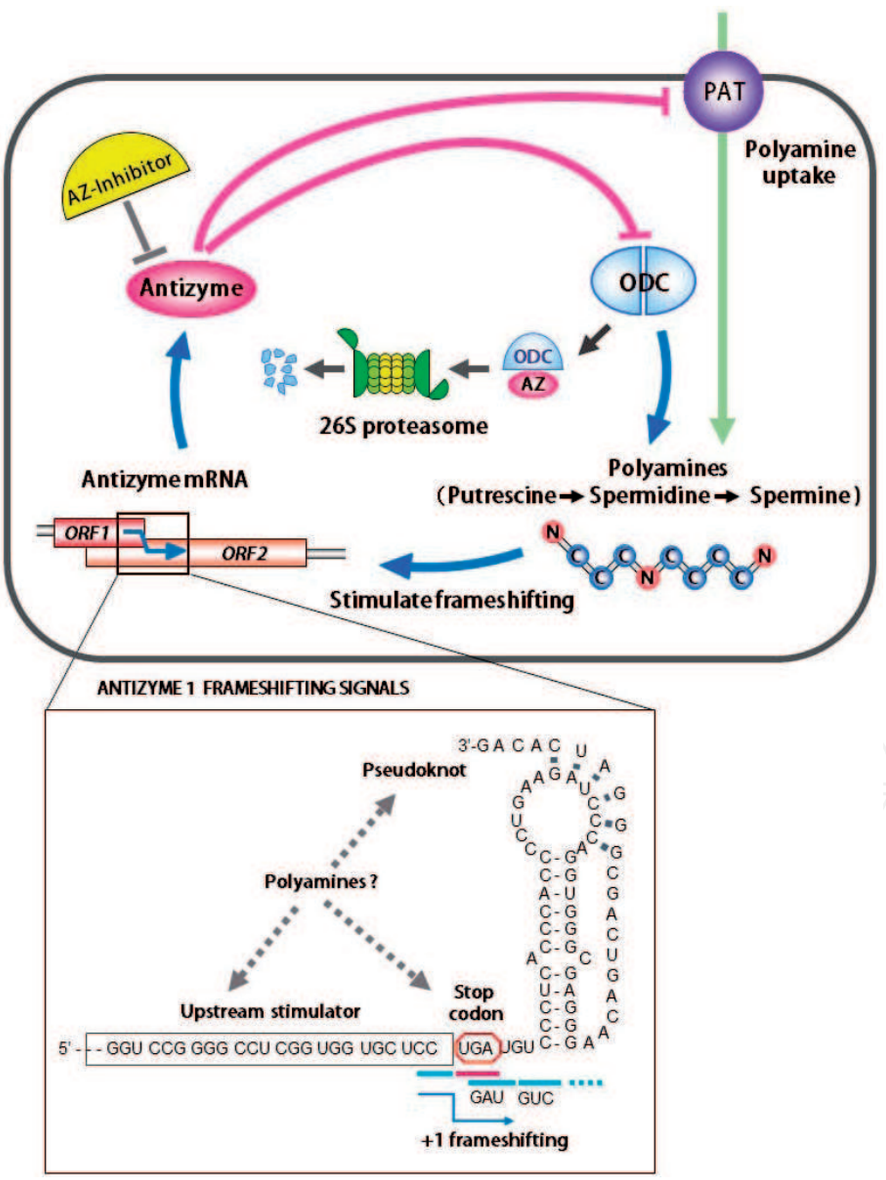

Figure 1.
Negative feedback regulation of cellular polyamine by antizyme. Three cis-acting elements, UGA stop codon, upstream stimulator, and pseudoknot structure, are known to be important for +1 frameshifting (bottom column). Putrescine, spermidine, and spermine are major polyamines in the mammalian cell. Putrescine synthesized from ornithine by ODC could be metabolized to spermidine and spermine in the cells. PAT is a polyamine transporter that uptakes polyamines from outside of the cells.

shifts +1 direction at the end of ORF1 (**Figure 1** bottom column). In this case, following ORF1, ORF2 is translated and full length active product "antizyme" is produced [12, 13]. The induced AZ protein binds to ornithine decarboxylase (ODC) monomer, a key enzyme in polyamine biosynthesis, and catalyzes the conversion from ornithine to putrescine and inhibits its activity. AZ-bound ODC is targeted to the 26S proteasome for degradation without ubiquitination (**Figure 1**) [14]. AZ also suppresses polyamine uptake by inhibiting membrane polyamine transporter (**Figure 1**) [15, 16]. Thus, AZ provides the negative feedback regulation of cellular polyamines. In addition, AZ is regulated by the protein, antizyme inhibitor (AZIN), that is homologous to ODC and can bind to AZ with higher affinity than ODC but lacking the enzymatic activity [17, 18].

In mammals, cells express three members of AZ protein family, AZ1–3 (**Table 1**) [19]. AZ1 and AZ2 are distributed ubiquitously in most of the tissues, whereas AZ3 is testis specific [20–22]. Both AZ1 and AZ2 bind to ODC and accelerate its

	AZ1	AZ2	AZ3
Species distribution	**Yeast-mammals**	**Vertebrates**	**Mammals**
Evolutional conservation	**Lower**	**Higher**	**Lower**
Tissue distribution	**Whole body**	**Whole body**	**Testis**
mRNA Expression	**High**	**Low**	**Low**
Cellular distribution	**Cytoplasm and Nucleus**	**Mainly nucleus**	**Spermatid and sperm**
Induction by polyamines	+	+	+
+1 frameshifting	+	+	+
ODC binding and inhibition	+	+	+
Acceleration of ODC degradation			
in vivo	+	+	-
in vitro	+	-	-
Inhibition of polyamine uptake	+	+	+
AZ inhibitor binding	+	+	+
Phenotype of knockout mice	**unreported**	**unreported**	**Male infertility**

Table 1.
Characteristics of antizyme family.

degradation in the cells [9, 23], but AZ3 has no activity for acceleration of ODC degradation [24]. The rate of ODC degradation by AZ1 is faster than that by AZ2 [23, 25]. Polyamine (putrescine) concentration of AZ1 knockdown cells is markedly increased, compared to that of AZ2 knockdown and control cells [26]. Therefore, it is thought that AZ1 mainly regulates cellular polyamine concentration. On the other hand, although AZ2 is highly homologous to AZ1 [25], it is considered that AZ2 is not a backup of AZ1 because of some differences between each other. AZ2 was found as one of the genes upregulated in neuronal cells by the drug that induces seizure [27]. Nucleic acid sequence of AZ2 is evolutionally conserved higher than that of AZ1 [11]. AZ2 is localized mainly in the nucleus [26] and is phosphorylated in the cells [28]. We will mention about AZ2 specific function with its interacting protein that we found very recently in this chapter.

3. Antizyme-interacting proteins and ubiquitin-independent proteasomal degradation

3.1 Antizyme 1-interacting proteins

It had been considered that ODC is the only protein degraded through AZ-mediated ubiquitin-independent proteasomal degradation system. However, recently several AZ1-interacting proteins other than ODC have been reported (**Table 2**). Although it has already been reported that those proteins are degraded by the ubiquitin-proteasome pathway, AZ1 could also accelerate those degradation without ubiquitination (**Tables 1** and **2**, **Figure 1**). Smad1, which is involved in bone morphogenetic protein (BMP) signaling pathway [29, 30], is the first reported protein that interacts AZ1 other than ODC [31]. In this case, newly synthesized HsN3, which is β-subunit for 20S proteasome, forms ternary complex with AZ1 and smad1. This complex may bind to 20S proteasome, and next 19S complex is docked on 20S, and then smad1 is degraded by the 26S proteasome.

Newman et al. reported that AZ1 has the ability to accelerate the degradation of cyclin D1, one of the cell cycle regulatory protein families [32]. Cyclin D1 interacts with cyclin-dependent kinase (CDK), and accumulation of cyclin D1-CDK complex is important for cell cycle progression [33]. This protein is already known to be degraded by ubiquitin-proteasome pathway [34]. They demonstrated that AZ1 induction by polyamine or overexpression of AZ1 accelerates cyclin D1 degradation, and knockdown of AZ1 suppresses it. Furthermore, in vitro experiment using purified cyclin D1, AZ1, and rabbit reticulocyte extracts as a source of 26S proteasome, AZ1 accelerated cyclin D1 degradation in a ATP-dependent manner. AZ1 could also degrade ubiquitin-deficient mutant of cyclin D1 in the cells [32]. In vitro size distribution analysis for binding between AZ1, cyclin D1, and ODC suggested that binding sites of AZ1 for cyclin D1 and ODC do not overlap each other, and cyclin D1 binds to the N-terminus of AZ1 and ODC binds at the C-terminus, respectively. Binding affinity of AZ1 to cyclin D1 is fourfold lower than that to ODC [35]. Although physiological significance is not clear, it showed that those three proteins form cyclin D1-AZ1-ODC ternary complex.

The oncogene Aurora A encodes a protein kinase that exerts essential roles in mitotic events and is important for induction of centrosome amplification [36]. Overexpression of Aurora A in many cancers induces aneuploidy, centrosome anomaly, poor prognosis, and invasiveness [37, 38]. Aurora A is ubiquitinated by the E3 ubiquitin (Ub) ligase, anaphase-promoting complex/cyclosome (APC/C) that is activated by both cell-division cycle protein 20 (Cdc20) and Cdh1,

Protein names	Protein function	Biological process	Characteristics of degradation
ODC	**Synthesis of putrescine from ornithine**	**Polyamine metabolism**	**AZ1 or AZ2 binds to ODC monomer that is targeted to the 26S proteasome [14, 23]**
Smad1	**Binding to smad4 Regulation of transcription**	**BMP signaling pathway**	**Formation of HsN3-Smad1-AZ1 ternary complex [31].**
CyclinD1	**Binding to CDK**	**Cell cycle**	**AZ1 binds to Cyclin D1 and accelerates its degradation In vitro and in vivo [32].**
Aurora A	**Protein Kinase**	**Mitotic events**	**Formation of AUKAIP-1-AZ1-Aurora-A ternary complex, and that is target to the proteasome in vivo [41].**
Msp1	**Protein Kinase**	**Cell divition**	**AZ1 affects the level of Msp1 at the centrosome [46].**
DNp73	**Inhibition of both p73 and p53**	**Apoptosis**	**c-Jundependent DNp73 degradation mediated by AZ1 in vivo [52].**
c-Myc	**Transcriptio n factor**	**Cell growth Differentiat ion survival Apoptosis**	**AZ2 binds to c-Myc and accelerates its degradation in vivo [26].**

Table 2.
The proteins degraded by antizyme-mediated ubiquitin-independent proteasomal pathway.

substrate-recognition subunit of APC/C, and is degraded by the proteasome [39, 40]. However, Lim and Gopalan demonstrated that AZ1 could accelerate Aurora A degradation with ubiquitin-independent manner, where Aurora A kinase-interacting protein 1 (AURKAIP1), a negative regulator of Aurora A, enhances the binding of AZ1 to Aurora A and facilitates the recognition of Aurora A by the proteasome [41].

Mps1 is protein kinase required for centrosome duplication in regulating the spindle assembly checkpoint [42, 43]. Accumulation of Mps1 at the centrosome causes aberrant centriole assembly [44, 45]. In fact in various tumor cells, centrosomal Mps1 pool is increased, which causes abnormal centrosome duplication [44]. Thus degradation of Mps1 is important for proper pool of Mps1 at the centrosome. Although degradation of Mps1 is known to be mediated by the proteasome, amino acid residue 420–507 of the human Mps1 that is sufficient for its degradation does not contain APC/C recognition motifs, suggesting the commitment of Mps1 to ubiquitin-independent proteasomal degradation [45]. Kasbek et al. reported that AZ1 localizes to the centrosomes and binds to Mps1 to control the levels of centrosomal Mps1 by accelerating the degradation of Mps1 [46]. Fluorescent microscopy analysis showed that centrosomal Mps1 level is dependent on AZ1 expression, overexpression of AZ1 decreases the centrosome Mps1 level, and conversely, AZ1 knockdown by siRNA increases that. Furthermore, deletion of degradation signal of Mps1 abolished the regulation of centrosomal Mps1 level by AZ1. In addition, overexpressing AZIN in the cells to trap AZ1 and inhibit its function increased centrosomal Mps1 level. Thus the balance of AZ1 and Mps1 level in the centrosome is important for the centrosome duplication process.

P73 is a homolog of p53 and exists as two major forms, TAp73 or Delta-N (DN) p73. TAp73 is full-length form and exerts proapoptotic function, whereas DNp73, which is amino-terminal transactivation domain lacking the form of p73, exhibits dominant-negative inhibitor activity for both p73 and p53, resulting in antiapoptotic properties [47]. Therefore, in the stress condition like DNA damage, reduction of DNp73 level is needed to execute apoptosis [48–50]. It is known that degradation of both TAp73 and DNp73 is mediated by E3-ubiquitin ligase Itch in a proteasome-dependent manner in normal condition [51]. However, in Itch-decreased condition such as DNA damage by UV irradiation, stabilization of TAp73 was observed, but DNp73 was not [51]. Therefore, it was considered that the degradation of TAp73 and DNp73 is regulated by different mechanisms. Dulloo et al. reported that reduction of DNp73 in the stress condition is due to the degradation of DNp73 by AZ1-mediated ubiquitin-independent proteasomal pathway [52]. They showed that degradation of DNp73 could be induced by genotoxic stresses such as UV irradiation and doxorubicin treatment. Inhibition of ubiquitin-activating enzyme E1 by the inhibitor PYR41 could not block DNp73 degradation, indicating that it relies on ubiquitin-independent pathway. They demonstrated that polyamine induced AZ1 to bind to DNp73 for accelerating its degradation. Interestingly, AZ1-mediated DNp73 degradation is dependent on transcription factor c-Jun that is activated by stress signals. Overexpression and knocking down of AZ1 also showed that even in the presence of c-Jun, AZ1 is necessary for genotoxic stress to induce DNp73 degradation. Although it is not clear how c-Jun operates AZ1 expression, c-Jun may act upstream of polyamine biosynthesis pathway.

Thus, several proteins degraded by AZ1-mediated proteasome pathway are found, but AZ2-interacting protein or AZ2-mediated proteasomal degradation other than ODC has not been reported. We recently found two AZ2-interacting proteins, and one of the two was the protein that accelerated its proteasomal degradation by AZ2 without ubiquitination (see next section).

3.2 Antizyme 2-interacting proteins

As mentioned above, AZ2 also binds to ODC and accelerates its degradation in the cells [9]. However, we have considered that AZ2 has specific function other than AZ1 because of the differences such as nuclear localization [26, 28], highly gene conservation between species [20], and high expression in neuronal cells [53].

We performed comprehensive analysis of AZ2-interacting protein using two-hybrid technique. Two AZ2-interacting proteins were identified. One is ATP citrate lyase (ACLY), which is the enzyme catalyzing acetyl-CoA production in cytosol [54] and related to lipid anabolism and acetylation of cellular components [55]. We found that ACLY binds not only to AZ2 but also to AZ1 by immunoprecipitation assay [56]. Degradation assay for ACLY was performed in expectation of ubiquitin-independent proteasomal degradation. However, AZs have no ability to accelerate ACLY degradation. Surprisingly, AZ1 and AZ2 activate catalytic activity of ACLY [56]. The other is proto-oncogene c-Myc that is a transcription factor with a basic region/helix–loop–helix/leucine zipper domain and forms heterodimer with Max for DNA binding [57, 58]. c-Myc functions as a master regulator of a variety of cellular processes such as cell growth, differentiation, survival, and apoptosis [58]. In cell growth, c-Myc targets ODC gene [59] and promotes synthesis of polyamine that is important for stabilization of nucleic acids, transcription, translation, and +1 frameshifting on AZ mRNA [6].

It is known that degradation of c-Myc is mediated by ubiquitin-proteasome pathway, where c-Myc is phosphorylated at Thr-58 (pT58) and Ser-62 (pS62) by extracellular signal-regulated kinase, ERK, and glycogen synthase kinase 3β, GSK-3β, respectively [60, 61]. After dephosphorylation at Ser-62 by protein phosphatase 2A, PP2A, pT58-c-Myc is ubiquitinated by E3-ubiquitin ligase Fbxw7 for proteasomal degradation [60, 62]. At first, AZ2-interacting protein identified by the comprehensive analysis mentioned above was not c-Myc but a protein that has basic region/helix–loop–helix/leucine zipper domain and interacts with c-Myc (Murai et al., manuscript in preparation). However, in the process of analyzing the interaction with AZ2, we found that AZ2 interacts with c-Myc in the cells by immunoprecipitation assay. Subcellular localization analysis of both proteins using fluorescent protein tags or antibody conjugated fluorescent probe revealed that AZ2 co-localized with

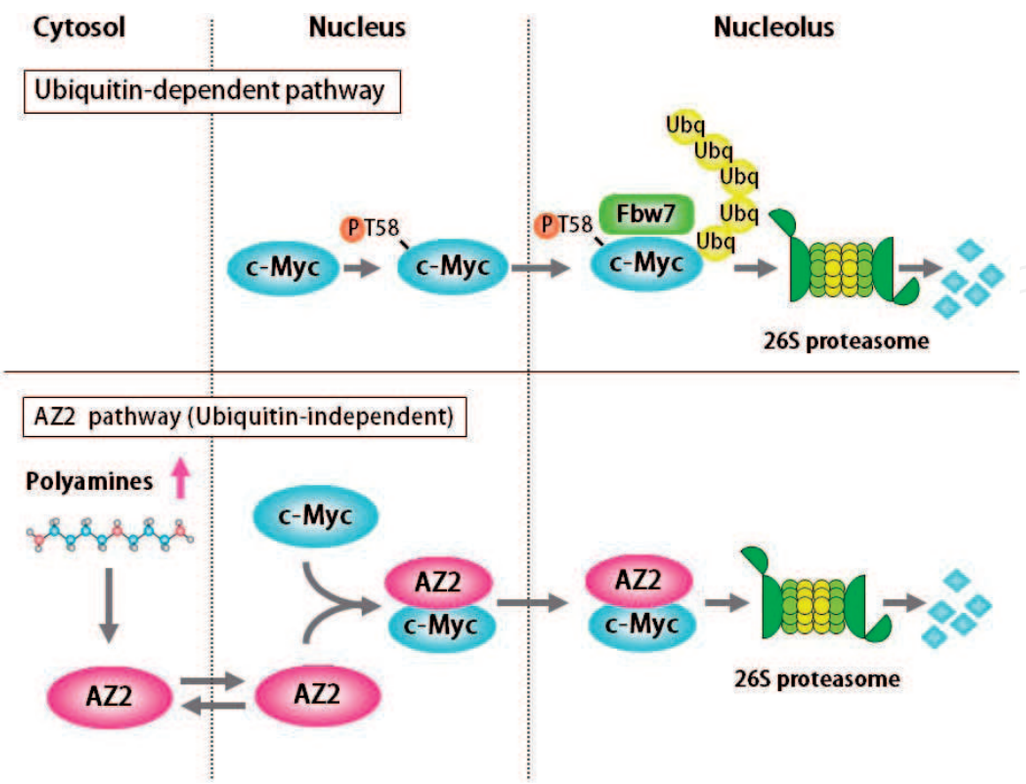

Figure 2.
AZ2-mediated c-MYC degradation in the nucleolus. Two distinct c-Myc degradation pathways exist in the cells. It is thought that AZ2 pathway functions under the stress condition (polyamine increased condition) such as glucose-free and hypoxia.

c-Myc in the nucleus. Treatment of proteasome inhibitor MG132 changes the nuclear co-localization of both proteins to nucleolar co-localization [26]. Overexpression of AZ2 or addition of polyamine in the cells accelerated c-Myc degradation, and knock-down of AZ2 with siRNA suppressed it. Furthermore, E1 inhibitor PYR-41 could not suppress AZ2-mediated proteasomal c-Myc degradation [26]. These results suggest that AZ2-mediated ubiquitin-independent nucleolar c-Myc degradation pathway other than ubiquitin-dependent one exists in the cells (**Figure 2**).

4. Conclusions

In this chapter, antizyme-mediated ubiquitin-independent proteasomal degradation has been discussed. All the proteins mentioned above are already known as the proteins degraded by ubiquitin-proteasomal pathway. It is not clear how antizyme-mediated ubiquitin-independent degradation of these proteins is physiologically significant. Normally subcellular localization of ODC is mainly in the cytoplasm and at least not in the nucleolus even in the presence of MG132. In addition, ODC is necessary for cell growth, and the affinity of interaction between antizyme and ODC is high [63]; in such condition, ODC probably occupies almost all antizymes in the cytosol, and antizymes hardly function for other antizyme-interacting proteins [64]. In this context, because subcellular localization of both AZ2 and its interacting protein c-Myc is in the nucleus or nucleolus, cytosolic protein ODC could not interact with AZ2 there. ODC is one of the c-Myc-targeting proteins, and AZ2 may function upstream of c-Myc especially under the stress condition such as glucose free and hypoxic condition [26]. Further studies are needed to elucidate the significance of antizyme-proteasome degradation pathway.

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