Microbiological Research 204 (2017) 9–29

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Review

Targets of ubiquitin like system in mycobacteria and related actinobacterial species

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ARTICLE INFO

Keywords:
Actinobacteria
Post-translational modification
Pupylation
Proteases
Prokaryotic ubiquitin-like protein
Mycobacterium

ABSTRACT

Protein turnover and recycling is a prerequisite in all living organisms to maintain normal cellular physiology. Many bacteria are proteasome deficient but they possess typical protease enzymes for carrying out protein turnover. However, several groups of actinobacteria such as mycobacteria harbor both proteasome and proteases. In these bacteria, for cellular protein turnover the target proteins undergo post-translational modification referred as pupylation in which a small protein Pup (prokaryotic ubiquitin-like protein) is tagged to the specific lysine residues of the target proteins and after that those target proteins undergo proteasomal degradation. Thus, Pup serves as a degradation signal, helps in directing proteins toward the bacterial proteasome for a turnover. Although the Pup–proteasome system has a multifaceted role in environmental stresses, pathogenicity and regulation of cellular signaling, but the fate of all types of pupylation such as mono and polypupylation on the proteins is still not completely understood. In this review, we present the mechanisms involved in the activation and conjugation of Pup to the target proteins, describing the structural sketch of pupylation and fundamental differences between the eukaryotic ubiquitin–proteasome and bacterial Pup–proteasome systems. We are also presenting a concise classification and cataloging of the complete battery of experimentally identified Pup-substrates from various species of actinobacteria.

1. Introduction: role of protein turnover in cellular physiology

Protein degradation is an omnipresent mechanism that maintains the cell integrity by eliminating damaged organelles, misfolded and damaged proteins from the cells and regulates various cellular processes related to them (Boubakri et al., 2015). Therefore, the living cells have multiple proteolytic systems to perform the degradation process and complex regulatory mechanisms to ensure that the proteolytic and synthetic processes are highly selective and thereby creating a balance between synthesis and breakdown so that the excessive breakdown of the cell constituents must be prevented (Lecker et al., 2006).

Many of the cellular proteins undergo protein recycling and are constantly being degraded to their constituent amino acids and replaced by newly synthesized ones (Hinkson and Elias, 2011). In eukaryotic cells, protein degradation is mediated by two major pathways namely, the lysosomal degradation pathway (LDP) and the ubiquitin-proteasome pathway (UPP) (Shang and Taylor, 2011). In the LDP, the majority of extracellular proteins and a few cell surface associated proteins are taken up by endocytosis or pinocytosis and thus degraded within the lysosomes whereas degradation of intracellular proteins is controlled *via* UPP (Chen and Ping Dou, 2010). UPP is one of the best-studied protein modification and degradation systems among eukaryotes that provide the recognition and specificity to target proteins for selective protein recycling (Weissman et al., 2011). In UPP, ubiquitin (Ub) conjugates to target proteins in a sophisticated multi-step activation and ligation pathway that eventually brings the doomed protein to the proteasome protease where they are targeted for proteolysis (Lorenz et al., 2013; Pal and Donato, 2014). UPP is an ATP-dependent process and has a diverse role during stress responses (Muralidharan and Mandrekar, 2013) and in many other biological processes including signal transduction and cell cycle regulation *via* cyclin degradation (Cajee et al., 2012), helps in the removal of misfolded or old and outdated housekeeping proteins and in cellular immune response through antigenic peptide processing (Myung et al., 2001).

2. Pupylation, protein turnover pathway in actinobacteria

Earlier studies in *Mycobacterium tuberculosis* (*Mtb*) (Pearce et al., 2008) and *M. smegmatis (Msm)* (Burns et al., 2009) revealed that like eukaryotes, these bacteria have Ub like the protein-tagging system to target proteins for proteolytic degradation. In such cases, the proteins

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http://dx.doi.org/10.1016/j.micres.2017.07.002
Received 25 May 2017; Received in revised form 22 June 2017; Accepted 5 July 2017
Available online 18 July 2017
0944-5013/

may be post-translationally modified by small, intrinsically disordered protein termed as Pup (Rv2111c) which are tagged as a monomer or in chains by a process known as pupylation (Imkamp et al., 2015; Bolten et al., 2017). Darwin and coworkers identified Pup has 69 kDa molecular weight and confirmed its interactions with their binding partners through biochemical analysis (Pearce et al., 2008). Pup-proteasome pathway (PPP) serves as a protective mechanism during stress conditions, such as carbon and nitrogen starvation (Elharar et al., 2014), oxidative stress (Compton et al., 2015), nitrosative stress (Darwin et al., 2003) and in the development of cell morphology (Boubakri et al., 2015) facilitating the pathogen to become resistant inside the host macrophages.

Similar to UPP, Pup covalently attaches through its C-terminus to the lysine side chains of substrate proteins *via* an isopeptide bond, ultimately destined them to the proteasome for degradation (Schrader et al., 2009; Burns et al., 2009; Samanovic et al., 2013; Striebel et al., 2014; Boubakri et al., 2015). This process involves three more proteins: PafA (Pup ligase), Mpa (Mycobacterial proteasomal ATPase) and Dop (Deamidase of Pup) (Iyer et al., 2008; Striebel et al., 2009; Chen et al., 2009). PafA acts as a regulatory core that directs the pathway of the bacterial PPP. It catalyzes the ligation of Pup to the substrate proteins (conjugation) and targeted them for proteasomal degradation. Interestingly, Pup also shows nonbonding interactions with Mpa which may probably help in the recognition of the degradation signal by the proteasomal ATPase (Liao et al., 2009; Striebel et al., 2009; Sutter et al., 2009). It was further reported that *mpa* gene is found in close proximity with *arc* (ATPase forming a ring-shaped complex) in other actinobacteria and in most cases, it is also localized in a separate operon upstream of the Dop encoding gene.

However, the Pup ligase gene, *pafA*, is located downstream of the proteasomal subunit genes and in some cases separated by multiple functionally unrelated open reading frames. In most of the actinobacterial species, the homologues of Pup, Dop, and PafA are present that are likely to be involved in pupylation (Burns and Darwin, 2010). Therefore, Pup may serve as a proteasomal recognition tag in these proteasome containing bacteria (Burns and Darwin, 2010; Barandun et al., 2012). We have concisely tabulated the commonalities and differences between the eukaryotic Ub-proteasome and prokaryotic Pup-proteasome systems as Table 1. In the following section, we are presenting molecular and structural characterization Pup protein carried out by us and others.

3. Molecular and structural characterization of pup protein

A helical wheel depiction of the conserved C-terminus (residues ranging from 37 to 60) of the homology modeled PUP (Rv2111c, from *Mtb*) was generated using software program Helical Wheel Projections (available at http://rzlab.ucr.edu/scripts/wheel/wheel) with default parameters. This tool was created by Don Armstrong and Raphael Zidovetzki at the University of California Irvine (Version Id: wheel.pl,v 1.4 2009-10-20 21:23:36 don Exp.) and used to visualize the distribution of hydrophilic and hydrophobic amino acids residues in α-helical protein structures around the central perpendicular axis. Fig. 1**a** showed that all the hydrophilic amino acid residues are found to be located on one face of the helix, whereas the hydrophobic amino acid residues are present on the other face. In this helix, the hydrophilic residues (N14, and Q24) are presented in red color circles while the hydrophobic residues (displayed as diamonds) are colored in different shades of green (L3, I7, L4, L11, Y22 and F18) at distinct faces of the helix (Fig. 1**a**). Besides that, the amino acid residues (A21, A15, V23, V19 and V10) coded in yellow colored circles represents the zero hydrophobicity. Potentially charged residues are displayed in triangles and pentagon shapes, highlighted in blue color. D1, D2, D5, D8, D9, D17, E6, E13 and E16 represent the negatively charged residues whereas R20 indicated the only positively charged amino acid residue (Fig. 1**a**). We have presented here, the cartoon representation of the Pup structure which makes the findings on distinct helical faces more profound (Fig. 1**b**). The protein sequence of pup from the Tuberculist Mycobacterium Information Database (Kapopoulou et al., 2011) was retrieved and was homology modelled using Phyre2 program (Kelley et al., 2015). The structure was visualized using PyMOL molecular graphics tool (DeLano, 2002). It was reported that the disordered amino terminus of Pup is required for degradation, while the helical carboxyl terminus may mediate the interaction with other proteins (Burns and Darwin, 2010; Geng et al., 2012). It is very likely that hydrophobic face of this C-terminal helix is involved in this interaction.

In *Mtb*, Pup is recognized by three pairs of tentacle-like N-terminal coiled coils of Mpa which translocates doomed proteins into the proteasome for destruction. Wang et al. (2010) reported the crystal structures of Mpa-Pup complex. Based on the crystal structure it was reported that the flexible C-terminal half of Pup comprising of amino acid residues 21-64 acquires a helical conformation upon binding to the N-terminal coiled-coils domains of Mpa that extend from the surface of the proteasomal ATPase ring to form a shared coiled-coil (Wang et al., 2010). Pup helix aligns with the Mpa coiled-coil in such a way that the flexible N-terminus of Pup repositions itself downward to the central channel of the hexameric ATPase, which may promote substrate entry into the pore of the Mpa and further translocation of Pup modifier together with the conjugated substrates, into the 20S proteolytic chamber. Whereas N-terminal half of Pup involved in unfolding and degradation of substrate proteins. Thus, Pup provides a two-part degrading apparatus to the proteasome substrates: the N-terminus of Pup is required for degradation and the portion that forms an α-helix mediates attachment to the Mpa (Wang et al., 2010). Using a combination of biochemical experiments and NMR methods, Sutter et al. (2009) also characterized the interaction of Pup with Mpa and showed that the residues of Pup ranging from 21–58 are responsible for binding to Mpa and that the coiled-coil domain of Mpa mediates this recognition. Studies showed that both hydrophobic and electrostatic interactions take place in between Pup and Mpa, and any disruption in each of the two interactions hinder Pup-mediated degradation by the proteasome in *Msm* (Samanovic et al., 2013).

Both PafA and Dop enzymes have a larger N-terminal domain (~400 residues) found homologous to the carboxylate-amine ligase family and consists of twisted central β-sheet packed against a cluster of helices, and a smaller C-terminal domain (~70 residues) unique to PafA and Dop members. The active sites of both enzymes are located on the concave surface of the β-sheet. A conserved groove leading into the active site could act as a spot where Pup binding takes place either in an extended or a helical conformation. Using NMR experiments and biochemical experiments, it was demonstrated that 30 amino acid residues located at the C-terminal of Pup showed interactions with its binding partners PafA and Dop (Özcelik et al., 2012; Burns and Darwin, 2010; Barandun et al., 2013).

Previous studies on Pup-PafA complex showed that Pup binds to a conserved groove on PafA (with a length of 40–50 Å) through a combination of hydrophobic interactions and salt bridges (Barandun et al., 2013). Upon binding to PafA, Pup undergoes a transition state from mostly disordered free state to a state with two well-resolved orthogonal helices H1 (38–47) and H2 (51–58) which are connected by a linker of three amino acids (E48–N50) (Barandun et al., 2013; Delley et al., 2017). Surprisingly, it was noticed that the helix H1 formed a four-helix bundle together with helices α8, α9, and α10 of PafA, anchoring Pup to the lower part of the Pup-binding groove (Barandun et al., 2013). It was observed that the 27 amino acid residues located at the C-terminal of Pup wrap around half of the PafA monomer to avoid an intramolecular attack by a lysine in the flexible N-terminal region of the Pup, which would be much faster than the intermolecular attack by the substrate lysine and could compete with substrate tagging. As Dop contains conserved surface residues in the H1 binding region, it could be possible that Dop could interact with Pup through similar binding mode. Özcelik et al. (2012) performed Gel-based *Mtb*PanB pupylation