Review

Diverse ubiquitin codes in the regulation of inflammatory signaling

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(Edited by Shigekazu NAGATA, M.J.A.)

Abstract: Ubiquitin is a small protein used for posttranslational modification and it regulates every aspect of biological functions. Through a three-step cascade of enzymatic action, ubiquitin is conjugated to a substrate. Because ubiquitin itself can be post-translationally modified, this small protein generates various ubiquitin codes and triggers differing regulation of biological functions. For example, ubiquitin itself can be ubiquitinated, phosphorylated, acetylated, or SUMOylated. Via the type three secretion system, some bacterial effectors also modify the ubiquitin system in host cells. This review describes the general concept of the ubiquitin system as well as the fundamental functions of ubiquitin in the regulation of cellular responses during inflammation and bacterial infection.

Keywords: ubiquitin, inflammation, TNF, bacterial effectors, autophagy

Introduction

Ubiquitin impacts its substrate’s fate by changing conformation, activity, and interactors.1) Ubiquitin is highly conserved from yeast to human, is ubiquitously expressed, and is used for posttranslational modification (PTM).2) Unlike other types of PTMs, including phosphorylation and acetylation, ubiquitin itself is often post-translationally modified, thus making it possible for ubiquitin to form a large number of ubiquitin codes. For example, ubiquitin can be ubiquitinated at 8 different sites, phosphorylated at 9 sites, and acetylated at 7 sites.3,4) Ubiquitination is not only used as a tag for proteasomal degradation but is also used as non-degradation tags in regulating cell signaling, cell cycle, DNA repair, autophagy, and cell death.1) All these different types of ubiquitination can distinguish the fate of a target substrate, thus contributing to various types of biological functions.

The choice of substrate and the ubiquitination type depend on the enzymes utilized for ubiquitination. In humans, there are two ubiquitin-activating E1 enzymes, ~40 ubiquitin-conjugating E2 enzymes, and ~600 ubiquitin E3 ligases. They catalyze ubiquitination by an ATP-dependent three-step action of E1-E2-E3.5) Although the precise biochemical properties of many of the E2s and E3s are yet to be understood, the variations in E2-E3 pairs seem to be very important.6) For example, the E2 enzyme UbcH5 functions with various types of E3 ligases whereas UbcH7 interacts with the homologous to the E6-AP carboxyl terminus (HECT) domain of HECT-type E3 ligases or the really interesting new gene (RING) 1 domain of RING-in-between-RING (RBR)-type E3 ligases.7) Such a pair determines the ubiquitination type and substrate.

A well-known cell signaling cascade, which involves several types of ubiquitination on multiple regulators, involves the tumor necrosis factor (TNF)-induced signaling cascade.8) Historically, ubiquitin-dependent proteasomal degradation of the ‘nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, alpha’ (IκB-α), which is an inhibitor or NF-κB, was first found as a ubiquitin-dependent critical process in this pathway.9) Later, non-degradation tags, specifically Lys63-linked ubiquitin chains, were found to be important to mediate signaling complex formation with ‘TGF-β activated kinase 1’ (MAP3K7) binding protein 2’ (TAB2)/‘TGF-β-activated kinase1’ (TAK1) to activate the downstream NF-κB signaling pathway.10) Now, we understand that more types of ubiquitin chains such as Lys11-linked chains11) and Met1-linked/linear
ubiquitin chains\textsuperscript{12–14} play a role in this signaling cascade. These atypical linkage types of ubiquitin chains are more recent members of the regulators of the TNF-induced signaling cascade.

Ubiquitination can be also induced by bacterial effectors when bacteria infect host cells.\textsuperscript{15} These bacterial effectors modify ubiquitin and substrates, which are different from the host side, thus making this PTM with more variations. There is also a defense mechanism against infection in some bacterial strains by autophagy where ubiquitin plays an important role.\textsuperscript{16} This review discusses the current state of knowledge on ubiquitin-dependent inflammatory cellular responses exemplified by the TNF-signaling cascade, bacterial effector functions, and selective autophagy for bacteria (xenophagy).

**How ubiquitination regulates cellular signaling cascades**

Ubiquitin regulates a wide variety of cellular signaling cascades. How can this ubiquitously expressed protein regulate different signaling cascades in a spatiotemporal manner? To answer this question, a few key features of ubiquitin should be pointed out. The first feature is the diversity of the ubiquitin code (Fig. 1a–c). Ubiquitin as a monomer can modify a substrate at a single site or at multiple sites. Because ubiquitination occurs also within itself, at 8 different sites, it can also form 8 linkage types of homotypic chains, which can be of different lengths (Fig. 1a).\textsuperscript{3} These 8 linkage types of ubiquitin chains, from a structural perspective, all look different from each other, making its modification style unique to each type. Furthermore, these linkage types of chains can be branched or mixed (Fig. 1b). Ubiquitin can be also phosphorylated, acetylated, and SUMOylated (Fig. 1c).\textsuperscript{3,4} These various types of ubiquitin chains modify a selected substrate to regulate cellular signaling cascades.

For ubiquitin chain formation on a selected site of a substrate, enzymes in the ubiquitin system play a critical role, which is the second important feature (Fig. 1d). For some linkage types of ubiquitin chains, E2s play a critical role. For example, cell division cycle 34 (CDC34) determines the linkage type of Lys48\textsuperscript{17,18} and the UbcH13/UEV1 pair determines the linkage type of Lys63.\textsuperscript{19} There is also another example where an E3 determines the linkage type.

![Fig. 1. (Color online) The ubiquitin system. (a) Various linkage types of homotypic ubiquitin chains (linked only via one site) linked via M1, K6, K11, K27, K29, K33, K48, or K63 located within a ubiquitin moiety formed on a model substrate. (b) Branched (upper panel) and mixed (lower panel) linkage types of ubiquitin chains formed on a model substrate. (c) Ubiquitin with or without posttranslational modifications (phosphorylation, acetylation, or SUMOylation) conjugated on a model substrate. (d) Enzymatic actions of ATP-dependent ubiquitination by a three-step cascade of an E1, an E2 and an E3, and deubiquitination by a deubiquitinase (DUB).](image-url)
The linear ubiquitin chain assembly complex (LUBAC), which is an E3 ligase complex, gives specificity to form Met1-linked/linear ubiquitin chains.\(^{20}\) However, it largely remains unclear which E2 or E3 gives specificity to a single linkage type of chains. To select a specific target substrate for ubiquitination, E3s are important, yet the functions of most of the predicted E3s remain open. Furthermore, enzymes hydrolyzing ubiquitin chains called deubiquitinases (DUBs) contribute to terminate ubiquitin-dependent signaling events.\(^{21}\) There are \(\sim100\) DUBs known so far, some of which have high specificity to ubiquitin chain-types, whereas some hydrolyze in a more promiscuous manner.\(^{21}\) Ubiquitin chain formation and destruction are controlled by these enzymes, thus how these enzymes are regulated is key for biological outcomes.

Once ubiquitin molecules as monomers or as chains are conjugated to a substrate, a new signaling complex forms by ubiquitin chain recognition via ubiquitin binding domains (UBDs).\(^{22}\) There are more than 150 proteins predicted to obtain a single or multiple UBDs, which may or may not have a specificity to a particular type of ubiquitin chains. As a matter of fact, many UBDs recognize a common I44 hydrophobic patch of a single ubiquitin moiety. However, there are also some UBDs that specifically recognize a particular type of ubiquitin chain.\(^{22}\) For example, there are proteins with UBDs called ‘ubiquitin binding in ABIN and NEMO proteins’ (UBAN), which specifically recognize Met1-linked/linear ubiquitin chains promoting the formation of signaling complexes to regulate the downstream signaling pathway.\(^{23}\) Proteins with such a UBD form a dedicated signaling complex in a spatio-temporal manner.

In summary, ubiquitin regulates cellular signaling via a) various types of ubiquitination of substrates, b) enzymes in the ubiquitin system leading to initiation and termination of the ubiquitination process, and c) UBD-containing proteins forming ubiquitin-dependent signaling complexes.

**The TNF signaling cascade includes the ubiquitin system**

Cellular signaling cascades where multiple types of ubiquitin chains play roles, are the inflammatory pathways. For example, the TNF-induced inflammatory signaling pathway is a well-known cascade, which is regulated by the ubiquitin system. TNF is an inflammatory cytokine and induces downstream cascades to activate kinases, such as the mitogen-activated protein kinases (MAPKs) including JNK, p38, and ERK and IκB kinase (IKK) in many different cell types.\(^{24}\) Among them, the NF-κB pathway activated via IKK is a notable example where ubiquitin plays a role.

Upon TNF binding to its receptor TNF receptor 1 (TNFR1) on the cell surface, the first signaling complex of the pathway, the TNFR complex I, is formed. Binding of trimeric TNF to trimeric TNFR1 leads to conformational changes of the cytosolic region of TNFR1, recruiting several proteins such as TNFRSF1A-associated via death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), cellular inhibitor of apoptosis protein 1 (cIAP1), A20, and receptor-interacting serine/threonine-protein kinase 1 (RIPK1) (Fig. 2). In this complex, cIAP1 is an E3 ligase, and A20 is a hybrid protein of DUB and E3 ligase. In this first step, RIPK1 is ubiquitinated with different linkage-types of ubiquitin chains including Met1, Lys11, Lys48, and Lys63.\(^{25}\) Depending on the linkage types of chains, the fate of RIPK1 differs. A notable example is A20-dependent ubiquitin chain editing on RIPK1. Because A20 has both DUB activity specific to Lys63-linked chains and E3 ligase activity to form Lys48-linked chains, A20 promotes RIPK1 for proteasomal degradation.\(^{26}\),\(^{27}\) RIPK1 is also important for regulating cell death pathways where caspase 8 cleavage occurs,\(^{28}\) showing multilayered regulation. Once ubiquitination occurs on RIPK1, LUBAC is recruited to the complex to linearly ubiquitinate RIPK1. Furthermore, the recruited IKK complex component NF-κB essential modulator (NEMO) is linearly ubiquitinated.\(^{14}\) LUBAC is the only known E3 ligase that can form Met1-linked/linear ubiquitin chains. All these events are important to degrade an inhibitor protein IκB-α in a ubiquitin-proteasome-dependent manner, which is initiated by IKK-dependent phosphorylation. This signaling cascade is a good example to understand that the editing of ubiquitin chains on a substrate by E3 ligases and DUBs plays an essential role in precisely regulating the downstream pathway. Regarding editing of the ubiquitin chains, it is known that the amount or length of ubiquitin chains also matters. For example, the LUBAC components, heme-oxidized IRP2 ubiquitin ligase-1L (HOIL-1L), HOIL-1L-interacting protein (HOIP), and SHANK-associated RH domain protein (SHARPIN) are all ubiquitinated.\(^{29}\),\(^{30}\) In this case, site-specific ubiquitination and the amount of ubiquitination influence downstream signaling complex formation, leading to regulation of NF-κB activation. DUBs such as
CYLD and OTU deubiquitinase with linear linkage specificity (OTULIN) also play an important role here by cleaving off linear ubiquitin chains to precisely edit these chains. It remains unclear why the TNF-induced NF-κB pathway involves so many ubiquitination events, and if there are any mechanistic reason for this.

Alternative ubiquitination signal by a third party, bacteria

Ubiquitin plays a critical role in inflammatory signaling cascades exemplified by the TNF pathway, but the modulators of ubiquitin do not necessarily derive from the host (Fig. 3a). NEMO is ubiquit}-
Ubiquitin regulates inflammatory responses

IpaH family members IpaH1.4 and IpaH2.5 ubiquitinate HOIP, and IpaH9.8 ubiquitinates NEMO both with K48-linked ubiquitin chains leading HOIP and NEMO for proteasomal degradation. In turn, inflammatory responses in host cells are suppressed. Similarly, IpaH4.5 ubiquitinates Rpn13 with K48-linked ubiquitin chains. Because Rpn13 is a proteasomal component, its degradation leads to dysfunction of the proteasome. Phosphoribosylation of ubiquitin induced by SdeA inhibits the ubiquitin system in host cells. This event can be inhibited by the deubiquitinase for PRubiquitination (Dup)A/B. (b) Autophagy targeting invading bacteria (e.g. Salmonella) involves the E3 ligase complex, LUBAC, leading to ubiquitination of bacterial proteins. Ubiquitin-coated bacteria are recognized by the autophagy adaptor optineurin (OPTN), which links them to autophagosomes. Using this selective autophagy towards bacterial (xenophagy), the cargo (bacteria) is finally degraded.

Fig. 3. (Color online) Ubiquitination upon bacterial infection. (a) Functions of bacterial effectors in host cells. The IpaH E3 family members, IpaH1.4 and IpaH2.5 ubiquitinate HOIP, and IpaH9.8 ubiquitinates NEMO both with K48-linked ubiquitin chains leading HOIP and NEMO for proteasomal degradation. In turn, inflammatory responses in host cells are suppressed. Similarly, IpaH4.5 ubiquitinates Rpn13 with K48-linked ubiquitin chains. Because Rpn13 is a proteasomal component, its degradation leads to dysfunction of the proteasome. Phosphoribosylation of ubiquitin induced by SdeA inhibits the ubiquitin system in host cells. This event can be inhibited by the deubiquitinase for PRubiquitination (Dup)A/B. (b) Autophagy targeting invading bacteria (e.g. Salmonella) involves the E3 ligase complex, LUBAC, leading to ubiquitination of bacterial proteins. Ubiquitin-coated bacteria are recognized by the autophagy adaptor optineurin (OPTN), which links them to autophagosomes. Using this selective autophagy towards bacterial (xenophagy), the cargo (bacteria) is finally degraded.

ubiquitated by LUBAC and other E3 ligases but also by IpaH9.8, a Shigella effector with E3 ligase activity. Upon ubiquitination by IpaH9.8, NEMO is degraded by proteasomes. Upstream of NEMO, IpaH1.4/2.5 effectors from Shigella ubiquitinate LUBAC for proteasomal degradation. In such ways, these effectors contribute to the immune responses in host cells, which restrict immune responses. The Shigella effectors can also control proteasome functions. IpaH4.5, another member of the E3 ligase family effectors, targets the proteasome regulatory particle 26S proteasome regulatory subunit RPN13’ for ubiquitination restricting proteasome-catalyzing peptide splicing. This means that in Shigella-infected
T cells, antigen-cross presentation to CD8-positive T cells via major histocompatibility complex (MHC) class I is reduced. A link between bacterial effectors and the ubiquitin system in host cells expands more towards DUBs.\textsuperscript{40} There are effector proteins from \textit{Shigella}, \textit{E. coli}, and \textit{Salmonella} with DUB activities with specificities for ubiquitin linkage types or for ubiquitin-like molecules.

\textit{Legionella} effector SdeA catalyzes an Arg residue in ubiquitin to form an ADP-ribose-ubiquitin intermediate, which can conjugate to a Ser residue in the substrate with phosphoribose ubiquitin (Fig. 3a). This event prevents activities of E1 and E2 in host cells, thus potentially inhibits many cellular events which depend on ubiquitin.\textsuperscript{41–44} Bacterial effectors with DUB functions specific to phosphoribose ubiquitin have been also identified.\textsuperscript{45,46} Thus, bacteria can control the functions of host cells through effector-dependent ubiquitination.

**A link to bacterial infection and autophagy**

There is also a pathway where ubiquitin controls infection by autophagy. Autophagy is a cellular process by which cellular garbage is removed. In cases with bacterial infection by \textit{Salmonella}, \textit{Listeria}, and \textit{Mycobacterium}, selective autophagy called xenophagy in host cells aims to remove these microbes.\textsuperscript{47} Here, the ubiquitin system also plays an important role. For selective targeting of cargos (\textit{e.g.} \textit{Salmonella}), cargos are ubiquitinated or ubiquitin-coated and then recruited to autophagosomes via an autophagy receptor, which has a ubiquitin-binding domain (Fig 3b). Autophagy receptors possess also a microtubule-associated protein 1 light chain 3 (LC3)-binding domain, which recognizes membrane-integrated LC3 family members on the autophagosome. It remained unclear whether all the known ubiquitin receptors have redundant functions to bridge a cargo and an autophagosome. More recent studies suggested distinct functions of these autophagy receptors regulating a specific type of selective autophagy for microbes (xenophagy) and mitochondria (mitophagy),\textsuperscript{48–51} which have protein domains recruiting other critical factors for autophagy in addition to these two binding domains for ubiquitin and LC3.

So, what are the ubiquitination types and enzymes regulating xenophagy? The entire picture remains to be seen, but there are some E3 ligases and suggested ubiquitination types for xenophagy regulation. LUBAC, which was discussed before as the regulator of NF-κB signaling, was also found to be an important regulator of xenophagy in \textit{Shigella}-infected cells.\textsuperscript{52} LUBAC generates Met1-linked/linear ubiquitin chains locally at \textit{Shigella}, recruiting OPTN to induce autophagy, as well as NEMO to activate NF-κB. Another E3 ligase LRSAM1 regulates xenophagy in \textit{Salmonella}-infected cells by ubiquitinating unidentified proteins on the bacterial surface.\textsuperscript{53}

Bacterial invasion on one hand activates machinery in host cells beneficial for the host, but on the other hand activates signaling in the host cells by bacterial effectors beneficial for the bacteria. Thus, both host cells and bacteria use the ubiquitin system in a way that each benefits from it. It is important to understand how these two systems compete, and to explore more details on the regulatory mechanisms of the ubiquitin system in host cells.

**Conclusion and perspective**

This review describes only the essential basics of how the ubiquitin system contributes to the regulation of inflammatory responses in cells. Since its discovery in 1975,\textsuperscript{54} the understanding of the ubiquitin system has expanded from proteasomal degradation of substrates to competing tools between the host and invading bacteria. Why this energy-consuming enzymatic action is so important in regulating every aspect of biology has always been of my personal interest. An assumption can be made that to maintain cellular homeostasis and to respond to any kind of stress, although complicated, precise control with a multi-layered regulatory step might be the only way. Notedly, it is not only within our own cells, but bacterial effectors directly modify ubiquitin and ubiquitination processes in our cells. Because there is no ubiquitination system in bacteria, they target only host cells using these effectors. We expect to encounter more surprises from the ubiquitin system in the future.

**Acknowledgements**

Research in the Ikeda lab is supported by JSPS KAKENHI Grant Number JP18K19959. I acknowledge all Ikeda lab members past and present.

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(Received Aug. 27, 2020; accepted Sep. 9, 2020)
Profile

Fumiyo Ikeda (born and raised in Osaka, Japan) graduated from the Dental School of Osaka University in 1999. She obtained a PhD degree from Osaka University in 2003 on the discovery of the transcription factor NFAT1 as a critical lineage determination factor during osteoclastogenesis. She continued as a postdoctoral fellow (supported by the Uehara Foundation, the Humboldt Foundation, and JSPS) in the Ivan Dikic lab at Goethe University (Frankfurt, Germany) where she studied a link between inflammatory responses and the ubiquitin system. During this period (2005–2011), she made a major contribution to the ubiquitin research field by the discovery of a new function of a novel type of ubiquitin chain in inflammatory cellular signaling. She served as an independent group leader at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA, Vienna, Austria) from 2011 to 2019, and moved to the Medical Institute of Bioregulation (MIB), Kyushu University, as a professor. Her team aims to understand how the ubiquitin system regulates inflammatory responses using various approaches, including biochemical, molecular, and cellular techniques as well as animal models. For her past achievements, she received the Dr. Paul und Cilli Weill Foundation Award in 2010 (Germany) and the JSPS Prize in 2019 (Japan).