Published in final edited form as:

Methods Mol Biol. 2014 ; 1209: 219–231. doi:10.1007/978-1-4939-1420-3_17.

Ubiquitination of Plant Immune Receptors

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Summary

Ubiquitin is a highly conserved regulatory protein consisting of 76 amino acids and ubiquitously expressed in all eukaryotic cells. The reversible ubiquitin conjugation to a wide variety of target proteins, a process known as ubiquitination or ubiquitylation, serves as one of the most important and prevalent post-translational modifications to regulate the myriad actions of protein cellular functions, including protein degradation, vesicle trafficking and subcellular localization. Protein ubiquitination is an ATP-dependent stepwise covalent attachment of one or more ubiquitin molecules to target proteins mediated by a hierarchical enzymatic cascade consisting of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase. The plant plasma membrane resident receptor-like kinase Flagellin Sensing 2 (FLS2) recognizes bacterial flagellin and initiates innate immune signaling to defend against pathogen attacks. We have recently shown that two plant U-box E3 ubiquitin-ligases PUB12 and PUB13 directly ubiquitinate FLS2 and promote flagellin-induced FLS2 degradation, which in turn attenuates FLS2 signaling to prevent excessive or prolonged activation of immune responses. Here, we use FLS2 as an example to describe a protocol for detection of protein ubiquitination in plant cells *in vivo* and in test tubes *in vitro*. In addition, we elaborate the approach to identify different types of ubiquitin linkages by using various lysine mutants of ubiquitin. The various *in vivo* and *in vitro* ubiquitination assays will provide researchers with the tools to address how ubiquitination regulates diverse cellular functions of target proteins.

Keywords

Ubiquitin; Ubiquitination; Polyubiquitin chain; Immunoblot; Immunoprecipitation (IP)

1. Introduction

Ubiquitin (originally named as ubiquitous immunopoietic polypeptide) is a highly phylogenetically conserved, 76-amino acid, 8.6 kDa protein that exists in all eukaryotic cells (1-3). One or more ubiquitin molecules could be covalently attached to a wide range of

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⁷Do not frequently freeze and thaw the 5 X UBX buffer as ATP will lose its activity.

proteins via its C-terminus or one of its 7 lysine (K) residues, which was known as ubiquitination or ubiquitylation (1, 4). Protein ubiquitination is catalyzed in an ATP-dependent manner by a stepwise enzymatic reaction cascade consisting of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase (1, 2, 5, 6) (Figure 1A). A single ubiquitin via its C-terminal glycine residue can be attached to a target protein, which is known as monoubiquitination (4). Monoubiquitination can occur at multiple lysine residues of a target protein, which is known as multi-monoubiquitination (4, 7). Further successive addition of many self-linked ubiquitin molecules to form a linear or branched ubiquitin chain is termed as polyubiquitination (5). Any of the seven lysine residues of ubiquitin could be used in the polyubiquitin chain synthesis, resulting in a highly complex chain (4). Notably, ubiquitination is a reversible process in the cells as many enzymes could cleave ubiquitin from mono-, multi-, and poly-ubiquitinated proteins (2, 3, 8).

Different types of ubiquitination modifications likely lead to distinct fates of the targeted proteins. Mono- or multi-mono-ubiquitination of cell surface-resident receptors often serves as a signal for receptor endocytosis and vesicle trafficking, which lead to either subsequent degradation in lysosomes, or recycling to the cell surface (1, 9, 10). For polyubiquitination, distinct polyubiquitin chains apparently play different roles in various signaling pathways (11). Two well-studied examples are the K48- and K63-linked chains (2, 12). The former type of polyubiquitin chain often directs the target proteins for proteasome-dependent proteolytic degradation (10), whereas the proteins tagged with K63-linked chain are often associated with DNA-damage responses and signaling processes, such as activation of the transcription factor NF- κ B (13). In addition, protein ubiquitination has been shown to modulate the protein subcellular dynamics (9). The unanchored polyubiquitination chains also appear to function as immune elicitors to activate innate immune signaling cascade (14). Thus, protein ubiquitination constitutes one of the key post-translational modification processes that modulate the myriad actions of protein cellular functions in diverse signaling pathways.

The plant plasma membrane resident receptor-like kinase (RLK) Flagellin Sensing 2 (FLS2) recognizes bacterial flagellin and functions as an immune receptor to initiate defense responses against pathogen attacks (15, 16). Upon flagellin perception, FLS2 instantaneously heterodimerizes with another RLK BAK1, which likely results in the subsequent phosphorylation of FLS2 and BAK1 complex (16, 17). The activated BAK1 phosphorylates a cytosolic kinase BIK1, which in turn leads to the release of BIK1 from FLS2/BAK1 complex to propagate intracellular immune responses (18). Once immune receptor is activated, it is essential to down-regulate immune signaling to prevent excessive or prolonged activation of immune responses. It has been observed that flagellin induces FLS2 translocation into intracellular vesicles, followed by degradation in a proteasome-dependent pathway (19). We have recently identified two plant U-box E3 ubiquitin ligases PUB12 and PUB13 that directly ubiquitinate FLS2 and promote flagellin-induced FLS2 degradation, which in turn attenuates FLS2 signaling (Figure 2). BAK1 directly phosphorylates PUB12 and PUB13 and is required for flagellin-induced FLS2-PUB12/13 association. Interestingly, FLS2 kinase activity and PEST domain, which are required for

internalization, are dispensable for PUB12/13-mediated FLS2 ubiquitination, suggesting different mechanisms or E3 ligases mediate FLS2 internalization and degradation (20). Here, we use FLS2 as an example to describe a protocol to detect protein ubiquitination *in vivo* in plant cells and *in vitro* in test tubes.

To establish an *in vivo* plant ubiquitination assay, we amplified UBQ10 from Col-0 cDNA library with polymerase chain reaction (PCR) and cloned it into a plant expression vector under the control of a constitutive Cauliflower Mosaic Virus 35S promoter. The UBQ10 was tagged with 2 X FLAG epitope at its N-terminus (FLAG-UBQ), whereas FLS2 was tagged with 2 X HA epitope at its C-terminus in a plant expression vector (FLS2-HA). The epitope tag of ubiquitin should be placed upstream of N-terminus of the open reading frame, leaving the C-terminus intact for subsequent isopeptide linkage to substrates. The epitope tag of FLS2 was placed at its C-terminus to avoid the potential influence of protein post-translational modifications signal at its N-terminus. The freshly isolated *Arabidopsis* mesophyll protoplasts were transfected with FLAG-UBQ and FLS2-HA and treated with specific elicitors such as flg22 (a conserved 22-amino acid peptide of flagellin). The ubiquitinated FLS2-HA proteins could be detected by anti-HA immuno-blotting after immunoprecipitation with anti-FLAG antibodies or anti-FLAG immuno-blotting after immunoprecipitation with anti-HA antibodies (Figure 3). The polyubiquitinated proteins are typically observed as strong smears or ladders with the molecular weight increase at about 8.6 kDa per band. In the case of the proteins with monoubiquitination modification, one extra band that is about 8.6 kDa bigger than the predicated size is often observed. Considering the abundance of endogenous ubiquitins which could also attach to FLS2 while are not able to be detected by anti-FLAG antibodies, the co-transfection of FLAG-UBQ with FLS2-HA greatly reduces the attachment of endogenous ubiquitins for the easy immunoprecipitation and immunoblotting with anti-HA or anti-FLAG antibodies. This assay does not require the knowledge of specific E3 ligases and is particularly sensitive to detect the dynamic changes of the target protein ubiquitination modification upon ligand treatment as it reveals all the potential ubiquitination modification events mediated by various endogenous E3 ligases to the target proteins. An *in vitro* ubiquitination assay could provide the direct evidence for the E3 ligase activity and the ubiquitination of target proteins. For *in vitro* assay that is optimized to detect *Arabidopsis* protein ubiquitination, we use *Arabidopsis* E1 (AtUBA1, AT2g30110) and E2 (AtUBC8, AT5g41700) that were purified from *E. coli* as 6xHis fusion protein. The AtUBC8 was chosen as an E2 because it has a broad activity towards different E3 ligases in *in vitro* ubiquitination assay (21, 22). In the case of FLS2 ubiquitination assay, we use glutathione S-transferase (GST)-fused PUB13 (AtPUB13, At3g46510) as an E3 and maltose binding protein (MBP)-fused FLS2 cytosolic domain (FLS2CD) as a substrate (Figures 1B & C) In addition, to explore the different types of ubiquitin linkage, we could perform the *in vivo* and *in vitro* ubiquitination assays with different versions of ubiquitin mutants that carry series of lysine (K) to Arginine (R) mutations. The poly-ubiquitin ladder would not be formed if the relevant ubiquitin lysine is mutated (Figure 4).

2. Materials

2.1 *In vivo* Ubiquitination Assay

1. FLAG-UBQ construct: Arabidopsis *UBQ10* gene tagged with 2X FLAG epitope at its N-terminus in a plant expression vector under the control of a constitutive Cauliflower Mosaic Virus 35S promoter.
2. Target gene constructs: PCR amplify the coding region of FLS2 or other target genes into a plant expression vector with 2X HA epitope at the C-terminus under the control of constitutive Cauliflower Mosaic Virus 35S promoter.
3. Protoplast isolation: refer the protocol reported by He et al. (23).
4. 2 mL round-bottom tubes.
5. Clinical centrifuge.
6. 40% (w/v) polyethylene glycol (PEG) solution: 40 % (w/v) of PEG4000, 0.1 M CaCl₂, and 0.2 M mannitol.
7. W5 solution: 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES pH 5.7.
8. MMg solution: 0.4 M mannitol, 15 mM MgCl₂, 4 mM MES pH 5.7.
9. WI solution: 0.5 M mannitol, 20 mM KCl, 4 mM MES pH 5.7.
10. Six-well tissue culture plates.
11. Flg22, the conserved 22-amino acid peptide of flagellin.

2.2 *In vitro* Ubiquitination Assay

1. 5 X UBQ buffer: 100 mM Tris-HCl pH 7.5, 25 mM MgCl₂, 2.5 mM DTT, 10 mM ATP.
2. Enzymes: His₆-AtUBA1 (E1, AT2g30110), His₆-AtUBC8 (E2, AT5g41700), GST-AtPUB13 (E3, AT3g46510), 1 µg/µL for each protein stored in 25% glycerol (v/v) solution, at -20°C (see Note 1).
3. MBP-FLS2CD-HA (cytosolic domain of FLS2 tagged with HA at its C terminus), 1 µg/µL stored in 25% (v/v) glycerol solution, at -20°C (see Note 1).
4. His₆-ubiquitin (BostonBiochem).
5. His₆-ubiquitin K to R mutants :
 - His₆-ubiquitin K6R (BostonBiochem)
 - His₆-ubiquitin K11R (BostonBiochem)
 - His₆-ubiquitin K27R (BostonBiochem)

¹N-terminal His₆-tagged AtUBA1 (E1) and AtUBC8 (E2) proteins were expressed in *Escherichia coli* and purified through standard nickel-agarose beads. N-terminal GST-tagged AtPUB13 protein was expressed from *E. coli* and purified through standard glutathione beads. Salts remained in the proteins will interfere with the *in vitro* ubiquitylation reaction. It is recommended to desalt the enzymes by flowing through the 30 KD centrifugal filters (Millipore). Proteins could be adjusted for storage with a concentration of up to 5 µg/µL at -80°C. Keep the proteins as small aliquots and avoid repetitive freeze-thaw cycles.

His₆-ubiquitin K29R (BostonBiochem)
His₆-ubiquitin K33R (BostonBiochem)
His₆-ubiquitin K48R (BostonBiochem)
His₆-ubiquitin K63R (BostonBiochem)
His₆-ubiquitin K6 only (BostonBiochem)
His₆-ubiquitin K11 only (BostonBiochem)
His₆-ubiquitin K27 only (BostonBiochem)
His₆-ubiquitin K29 only (BostonBiochem)
His₆-ubiquitin K33 only (BostonBiochem)
His₆-ubiquitin K48 only (BostonBiochem)
His₆-ubiquitin K63 only (BostonBiochem)
His₆-ubiquitin no lysines K0 (BostonBiochem)

2.3 Immunoprecipitation and Immunoblot

1. 4X SDS-PAGE sample buffer: 0.2 M Tris-HCl pH 6.8, 80 g/L SDS, 40% (v/v) glycerol, 50 mM EDTA pH 8.0, 0.8 g/L Bromophenol Blue, and 4% (v/v) β -Mercaptoethanol.
2. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), containing 7.5% (w/v) polyacrylamide-co-methylene-bis-acrylamide.
3. Antibody: anti-HA-peroxidase (HRP), anti-FLAG-M2 peroxidase (HRP) (Sigma), , anti-ubiquitin, anti-Rabbit IgG-peroxidase (HRP), anti-FLAG M2 affinity gel (Sigma).
4. Immunoblot PVDF membrane for protein blotting.
5. Immunoprecipitation buffer (IP buffer): 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA pH 8.0, 1% (v/v) Triton X100, 1 mM DTT, 2mM NaF, 2mM Na₃VO₄, 0.5% (v/v) protease inhibitor cocktail.
6. 50 mM Tris-HCl pH 7.5.
7. SDS-PAGE running buffer: 3 g/L Tris base and 14.4 g/L glycine and 1 g/L SDS.
8. Transfer buffer: 3 g/L Tris base, 14.4 g/L glycine, 20 % (v/v) methanol and 0.1 g/L SDS, final pH 8.3.
9. Phosphate-buffered saline (PBS) buffer: 8 g/L NaCl, 0.2 g/L KCl, 2.14 g/L Na₂HPO₄, and 0.2 g/L KH₂PO₄.
10. PBST buffer: add 0.1 % (v/v) Tween-20 into PBS solution before use.
11. Blocking solution: 5% (w/v) milk dissolved in PBST solution.
12. Supersignal west pico chemiluminescent substrate (Thermo).

3. Methods

3.1. *In vivo* Ubiquitination Assay

1. Add 50 μ L of the mixed FLAG-UBQ and FLS2-HA DNA with the concentration of 1.8 μ g/ μ L into a 2 mL round-bottom tube (see Note 2).
2. Add 500 μ L of protoplasts with the density of 2×10^5 cells/mL suspended in MMg solution (see Note 3).
3. After adding protoplasts, immediately add 550 μ L of freshly prepared 40% PEG, gently mix the DNA and protoplasts by tip-taping the tube.
4. Incubate at room temperature (23°C) for 8~10 min.
5. Add 1.5 mL of W5 solution and mix well to stop the transfection.
6. Spin at 100 g with a clinical centrifuge for 1 min and suck out the solution without disturbing the protoplast pellets.
7. Resuspend the protoplasts in 100 μ L of WI solution gently.
8. Transfer the protoplasts into a six-well tissue culture plate with 1 mL of WI (see Note 4).
9. Incubate the protoplasts 8 to 12 hr at the room temperature (23°C).
10. Add flg22 to a final concentration of 1 μ M and incubate 30 min at the room temperature.
11. Harvest the protoplasts by centrifugation at 100g for 1 min and suck out the supernatant.
12. Add 300 μ L of IP buffer, vortex for 5~10 min and chill on ice for 5 min.
13. Remove the cellular debris by centrifugation at 13,000 rpm for 5 min at 4°C. Keep the supernatant for the following immunoprecipitation (IP).
14. Take out 15 μ L of the IP samples, add 5 μ L of 4 X SDS sample loading buffer, heat at 95°C for 5min, and store as protein loading controls.
15. Add 20 μ L of anti-FLAG agarose into the remaining IP samples (see Note 5).
16. Incubate at 4°C for 2~4 hr on a rocker.
17. Spin down the agarose beads at 100g for 1 min, suck out the supernatant completely.

²The quality of DNA is critical for protoplast transfection. Low quality DNA could result in reduced protein expression level or even dead protoplasts. It is highly recommended to use CsCl gradients for plasmid DNA isolation. The ratio of FLAG-UBQ and the target DNAs can vary from 2:1 to 1:2, depending on the target protein expression level.

³The amount of protoplasts used for transfection can be scaled up or down with the same DNA/protoplast ratio depending on the target protein expression and ubiquitination level and the purpose of individual experiments. A large scale of protoplasts, such as 10~20 ml with a concentration of 2×10^5 cells/mL, can be used to express proteins for mass spectrometry analysis of protein ubiquitination sites and chain linkages.

⁴To prevent sticking of protoplasts to the bottom of the plates, the plates can be pretreated with 5% calf serum for 1 sec before use.

⁵Anti-FLAG agarose needs to be pre-washed with IP buffer to remove storage solution which may interfere with protein binding.

18. Wash the agarose beads with 500 μ L ice-cold IP buffer (see Note 6).
19. Repeat *step 17* and *18*.
20. Wash one more time with 500 μ L ice-cold 50 mM Tris-HCl pH 7.5, and suck out the supernatant completely.
21. Add 5 μ L of 4X SDS-PAGE sample buffer and heat at 95°C for 10 min.
22. Store the samples at –20°C until ready for immunoblotting.

3.2. *In vitro* Ubiquitination Assay

1. Prepare each reaction in a 1.5 ml tube by mixing the following:

1 μ g	E1
1 μ g	E2
1 μ g	E3
1 μ g	ubiquitin or various ubiquitin mutants
0.5 μ g	substrate protein tagged with HA epitope
6 μ L	5 X UBQ buffer (see Note 7)
add water to a final volume of 30 μ L	

No E1 is added in the reaction as a negative control (Figure 4B) (see Note 8).

2. Spin down at 250 g. Incubate the reaction tubes on a roller at 100 rpm for 2 hr at 28°C (see Note 9).
3. Add 10 μ L of 4X SDS-PAGE sample buffer and heat the samples at 95°C for 5 min to stop the reaction.
4. Freeze and store the samples at –20°C until ready for immunoblotting.

3.3. Immunoblot Detection

1. Load 10 μ L of the reaction samples from *in vivo* or *in vitro* ubiquitination assays onto a 7.5% acrylamide SDS-PAGE gel. Include pre-stained protein molecular weight standards in one lane as reference to estimate the molecular weight of the target proteins.
2. Load the same samples to another SDS-PAGE as *step 1*. Both gels are treated exactly the same from *step 3* to *step 6*.

⁶At least 500 μ L IP buffer is recommended for each wash, and any trace amount of supernatant should be completely removed with a fine tip. This will reduce the nonspecific protein contamination. DTT, NaF, Na₃VO₄, and protease inhibitor cocktail can be eliminated in the IP washing buffer.

⁸In addition to wild-type ubiquitin protein, His₆-ubiquitin lysine mutants can be used to identify the type of polyubiquitin chains (Figures 4A & B). For instance, if only K48 is used for polyubiquitin chain synthesis, ubiquitin K48R mutation will block polyubiquitination and the ladder collapses to a monoubiquitination band (Figure 4C). At the same time, among the single lysine ubiquitin proteins, ubiquitin K48, but not the others, is competent for the polyubiquitin chain synthesis shown as a ladder in the corresponding lane (Figure 4D).

⁹The incubation time could be extended up to 12 hr to enhance protein ubiquitination efficiency. However, the prolonged incubation of proteins at 28°C may affect protein stability. Typically, incubation times no longer than 12 hr are recommended.

3. Use a constant voltage of 80 V for 20~40 min until the protein dyes reach the resolving gel, and run another 1~2 hr at 150 V until the target proteins approach the bottom of the gel (can be estimated according to the predicated molecular weight).
4. Fully wet the PVDF membranes by immersion in methanol for 30 second, and then in transfer buffer for 15~30 min to facilitate the binding of ubiquitinated proteins to the membrane.
5. Electro-transfer proteins from the SDS-PAGE to a PVDF membrane at the constant current of 200 mA for 2~3 hr in transfer buffer (see Note 10).
6. Incubate the membrane in a small tray with blocking buffer at room temperature for 1 hr on an orbital rocker. Alternatively, membrane can be blocked overnight in a cold room.
7. Incubate one membrane with anti-HA-HRP antibodies for 2 hr or longer on an orbital rocker in a cold room (see Note 11).
8. Incubate the other membrane with anti-ubiquitin antibody. All the ubiquitinated proteins, including E1, E2, E3 and substrate proteins could be potentially detected by anti-ubiquitin antibody. Strong smears are often seen (*step 12*), indicating a successful ubiquitination reaction (Figure 1C). Since the ubiquitin in the *in vivo* ubiquitin assay is tagged with FLAG epitope, anti-FLAG-HRP antibody can also be used to assess the reaction efficiency (Figure 3).
9. Wash the membrane with PBST buffer for 3 times, 10 min for each time.
10. If anti-ubiquitin antibody is used in *step 8*, incubate the membrane with anti-rabbit IgG-HRP secondary antibody with a dilution of 1:5000 in blocking buffer for 30~60 min at room temperature and then repeat *step 9*. For the membrane that is incubated with a HRP-conjugated primary antibody, *step 10* should be skipped (see Note 12).
11. Immerse the membrane in a small volume of “supersignal west pico chemiluminescent substrate”. After 1 min, immediately seal the membrane inside a small transparent plastic bag (see Note 13).
12. Stick the membrane to the inside of a cassette and expose the membrane to a film in the dark room.

¹⁰Both “semi-dry” and “wet transfer” are acceptable, but the wet transfer tends to have relatively higher transfer efficiency, especially for high molecular weight proteins. So we recommend to transfer ubiquitinated proteins with “wet transfer” method described by Towbin (24). Moreover, autoclaving the membranes in deionized water for 20 min will denature the ubiquitinated proteins, thereby increasing the reactivity to anti-ubiquitin antibodies, some of which only target denatured ubiquitins. Heat activation may not be applied to all anti-ubiquitin antibodies (25).

¹¹Antibody can be diluted in blocking buffer at a ratio of 1:2000~1:5000. For the *in vitro* ubiquitination assay, the incubation time of a primary antibody can be as short as 30 min.

¹²Secondary antibody can be diluted in blocking buffer at a ratio of 1:5000 ~ 1:10000. High concentration and too long incubation time may enhance the background.

¹³“Supersignal west femto chemiluminescent substrate” (Thermo) can be used to enhance immunoblot signal. We recommend to mix “Supersignal west femto chemiluminescent substrate” with “Supersignal west pico chemiluminescent substrate” (Thermo) at a ratio of no higher than 1:10 to avoid too strong bleaching signal.

Acknowledgement

We thank Dr. Dongping Lu for the initial work to establish various ubiquitination assays. The work was supported by the funds from NIH R01GM092893 to P.H and R01GM097247 to L.S.

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Figure 1. *In vitro* ubiquitination assay
(A) Protein ubiquitination is catalyzed in an ATP-dependent manner by hierarchical stepwise enzymatic reactions consisting of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin-ligase. (B) In an *in vitro* ubiquitination assay, the substrate proteins are mixed with E1, E2, E3, ubiquitin and ATP in a 1.5 ml tube for a reaction and subjected for an immuno-blot with an antibody specifically recognizing the substrates. The ubiquitinated proteins are typically observed as strong smears or ladders with the molecular weight increase at about 8.6 kDa per band. (C) Immunoblot of ubiquitinated proteins. Ubiquitinated FLS2-HA was detected by anti-HA immunoblot, total ubiquitinated proteins were shown in anti-ubiquitin immunoblot.

Figure 2. Activation and attenuation of FLS2 signaling

Flagellin induces complex formation and transphosphorylation to activate FLS2 signaling and immune responses. The activated PUB12/13 ubiquitinate FLS2 leading to its degradation and attenuate immune signaling.

Figure 3. *In vivo* ubiquitination of FLS2 assay

The *Arabidopsis* protoplasts co-transfected with FLAG-UBQ and FLS2-HA were treated with 1 μ M flg22 for 30 min. The ubiquitinated FLS2-HA proteins could be detected by anti-HA immunoblot after immunoprecipitation (IP) with anti-FLAG antibodies.

Figure 4. Identification of the types of polyubiquitination chains

(A) K to R point mutants of ubiquitin. Only K residues of ubiquitin are shown and the numbers indicate the positions of Ks in ubiquitin. Each mutant carries a K to R mutation in an indicated position. (B) Single K mutants of ubiquitin. All the K residues are mutated into Rs except the indicated positions. (C) and (D) Identification of polyubiquitin chain structure *in vitro*. Here depicts only one type of the polyubiquitin chains. In the case shown here, only K48 of ubiquitin is involved in chain synthesis. Therefore, K48R ubiquitin cannot be used as a substrate (C), while among the single-lysine ubiquitins, only K48-ubiquitin is compatible for the polyubiquitin chain synthesis (D).