Role of ubiquitin-like proteins in cellular function

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There are at least five ubiquitin homologues in mammals, named as Nedd8, SUMO-1, Smt3a, Smt3b, UCDRP and Elongin B. The Nedd8 is a modifier of cullin family and this modification is catalysed by APPBP1/Uba3 as E1 and Ubc12 as E2. Recently, the cullin1 in SCF is detected to be modified by Nedd8. Also cullin2 in VCB, another E3 complex, is modified by it. More recently, cullin 1 to 4 have been found to be modified by it. The modified cullin is mainly found in centrosome, and also the ratio of the modified cullin increases when the cullin is expressed together with Roc1 in Sf-9 cells in baculovirus protein expression system. However, the role of this modification has not been cleared yet.

Today I'm going to present you that Nedd8 modification of cullin1 is necessary for activity of SCF *in vitro* and *in vivo*.

Another ubiquitin like protein, SUMO-1/sentrin / PIC1 also covalently binds to target proteins as ubiquitin does. As target proteins for this modification, RanGAP1, PML, Sp100, IκBα, and so on have been detected. This post-translational modification could result in a change in the intracellular localization and inhibition of ubiquitination of the target proteins.

I'm going to present that this modification occurs in three sites of the C-terminal region of p53, the transactivation regulatory domain of it, one of which is also the acetylation site by p300/CBP.

(I) Nedd8 modification of cullin1 is necessary for activity of SCF *in vitro* and *in vivo*.

Ubiquitination of p27 by SCF(skp2) in the presence of Nedd8 modification.

The Roc1 protein is found as binding protein of cullin family proteins and activate the ubiquitination of IκBα by SCF(TRCβ). To know whether the Roc 1 protein is also important in the activity of SCF(skp)2 toward p27, the ubiquitination assay was carried out in the presence or absence of Roc1 protein. The ubiqutination of p27 increased in the presence of Roc1. To know whether the Nedd8 modification is important for the activity of SCF, the ubiquitination of p27 was carried out with or without Nedd8 modification system, APPBP1/Uba3, Uba12, and Nedd8. The ubiquitination of p27 was markedly increased in the presence of Nedd8 modification system.

Effect of Nedd8 un-modified mutant cullin 1 on SCFskp2 activity

The mammalian cullin2 is modified at K689 by Nedd8. When human cullin1 is aligned with cullin2, Nedd8 modification site cullin2 is correspondent to K696 in cullin1. The K696 residue was modified to R696 and the Nedd8 modification of this mutant cullin1 was tested. After the wild or mutant cullin1 was subjected to the Nedd8 modification system, the modification of it was checked. By western blotting using anti-Nedd8 antibody, the modification of wild cullin1 was detected, but that of mutant cullin 1 was not. After Nedd8 modification reaction was carried out in the presence of wild or mutant cullin1, the ubiquitination of p27 was compared. The SCF containing mutant cullin1 did not show any activity of ubiquitin ligase toward p27.

Ubc12 dominant negative mutant inhibits the degradation of IκBα in a cell.

C111A mutant of Ubc12 does not have the activity of Nedd8 modification of cullin1. When HeLa cells was treated with TNFα, NκB in a cytoplasm was translocated to nucleus as a result of degradation of IκBα through ubiquitin-proteasome pathway. On the other hand, when the cells were transfected with dominant negative mutant of Ubc12 before the treatment with TNFα, the translocation of NFκB into nucleus did not occur. The degradation of IκBα was not stimulated by the treatment with TNFα. This means that Nedd8 modification of cullin1 is necessary step of degradation of

IκBα.

(II) SUMO-1 modification of p53 at carboxyl terminal region enhances the transactivation activity of p53.

SUMO-1 conjugation needs two enzymatic Steps, E1 and E2.

We isolated mammalian SUMO-1 conjugation enzyme E1. Sua1/hUba2 heterodimer is an E1 enzyme for SUMO-1 modification, and Sua1 and hUba2 have a similarity to the N- and C-terminal region, respectively, of ubiquitin E1. E3 enzyme is not required for SUMO-1 modification, so Ubc9 (E2) recognizes target protein and transfers the SUMO-1 molecule to the protein.

SUMO-1 modification of p53 occured *in vitro* and *in vivo*.

HeLa S3 cells were transfected with p53, p53 and Flag-SUMO-1, or p53 and Flag-Flag-SUMO-1 cDNAs in order to test the possibility of covalent modification of p53 with SUMO-1 *in vivo*. Lysates were prepared at 16 h after transfection, and p53 protein was detected by immunoblotting using anti-p53 antibody (DO-1). We detected a high-molecular-weight form of p53, which included the additional molecular weight (12K) of SUMO-1, in a SUMO-1-dependent manner. In the presence of Flag-Flag-SUMO-1, the modified p53 showed much slower migration than in the presence of Flag-SUMO-1. We next examined the modification of cellular p53 by expressing Flag-SUMO-1 in 293 cells. SUMO-1 molecule formed a covalent bond to endogenous p53 protein, too . These data indicate that p53 is covalently modified by SUMO-1 in intact cell. To support our *in vivo* data, we tested whether p53 could be modified by SUMO-1 *in vitro*. We detected SUMO-1 conjugation to p53 in the presence of Sua1/hUba2 complex (E1) and Ubc9 (E2).

p53 is modified by SUMO-1 at three sites in the carboxyl terminal domain

p53 has three functional domains, N-terminal, core and C-terminal domain, and post-translational modification (e.g., phosphorylation and acetylation) is focused on N- and C-terminal regions. Substitution of Lys for Ala

or Arg in the C-terminal region absolutely inhibited conjugation of SUMO-1 to p53 (K370-386A and K370-386R). K370/372/373R was able to form a covalent bond with SUMO-1, whereas K381/382/386R could not. We then examined single mutants of Lys 381, 382, or 386; however, all the mutants were modified by SUMO-1. As both K381/386R and K382/386R failed to bind covalently, this suggest that Lys 381, 382, and 386 are involved in the modification of p53 by SUMO-1. Actually, the transfectant K381/382/386R was not modified by SUMO-1 in HeLa cells.

Transactivation activity of p53 is enhanced by SUMO-1 modification.

As p300/CBP acetylates p53 *in vivo* and *in vitro* and activates DNA-binding activity of p53 through its acetylation, we performed an acetylation assay using p53 mutants. The mutants with a substitution at Lys 382 (K370-386R and K381, 382, 386R) were acetylated less than the wild-type p53 and at the same level as K382R. Thus, p300/CBP acetylated p53 mainly on Lys 382, which was involved in SUMO-1 modification. When p53-/- MEF was transfected with p53, a reporter plasmid which has p53 responsible elements and luciferase gene, and with or without SUMO-1, the luciferase activity of the cells transfected with SUMO-1 was higher than that of the cells without SUMO-1 transfection. Furthermore, when the cells were transfected with p53 mutant, K381, 382, 386R, the activity was much higher than that of the cells transfected with wild type p53.

In this presentation, I will also present the recent results about the SUMO-1 modification of MDM2, ubiquitin ligase for p53.