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The mechanism of poly-NEDD8 chain formation in vitro

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ABSTRACT

NEDD8 is a small ubiquitin-like protein that modifies target proteins in a reaction similar to ubiquitination. In this reaction, three enzymes are required and sufficient: NEDD8 activating E1-like enzyme (APP-BP1/Uba3), NEDD8-specific E2 enzyme (Ubc12) and RING-finger protein ROC1 (NEDD8 E3 ligase). Unlike ubiquitin, which is well known to form poly-ubiquitin chain, little is known about the formation of poly-NEDD8 chain. Here we show the mechanism of poly-NEDD8 chain formation on Cullin-1 using a complete in vitro reconstituted NEDD8 conjugation system. Intriguingly, poly-NEDD8 chain was build up on catalytic Cysteine residue of Ubc12. Furthermore, Ubc12 formed poly-NEDD8 chain without the activity of ROC1. Rather ROC1 mutant, defective for ubiquitin ligase activity, dramatically enhanced the poly-NEDD8 chain formation. In turn, ROC1 was essential for the transfer of poly-NEDD8 chain from Ubc12 to Cul-1. These results suggest the important regulatory role of ROC1 for poly-NEDD8 chain formation.

Introduction

NEDD8 is a ubiquitin-like modifier that is evolutionarily conserved and most similar with ubiquitin [1,2]. NEDD8 conjugates to target proteins via a three-step enzymatic pathway analogous to that of ubiquitin [3–5]. In the first step, NEDD8-specific E1-like enzyme, APP-BP1/Uba3 heterodimer, activates NEDD8 by adenylating the carboxyl terminal glycine residue of NEDD8 followed by formation of high-energy thioester bond with catalytic Cysteine residue of Uba3. In the next step, NEDD8 is transferred from E1 to catalytic Cysteine residue of NEDD8-specific E2 enzyme Ubc12 in the same thioester linkage. Finally, active NEDD8 charged on Ubc12 is subsequently transferred and conjugated to the substrates with the help of E3 ligases. The major substrates of NEDD8 are Cullin Ring Ligases (CRLs) [6]. ROC1 subunit in CRLs interacts with Ubc12, and functions as NEDD8 E3 ligase for Cullins.

Recently, it was reported that stably expressed NEDD8 in the cells forms a poly-NEDD8 chain by conjugating to its own Lysine residues [7,8]. However, target substrates of poly-NEDD8 chain and the mechanism by which poly-NEDD8 chain is formed are yet to be determined. In the present study, we devised a complete in vitro NEDD8 conjugation system solely made by recombinant proteins purified from *Escherichia coli* and reconstituted the poly-NEDD8 chain formation on Cullin-1. The results suggest the importance of ROC1 activity for poly-NEDD8 chain formation.

Materials and methods

Plasmids and protein purifications. Mouse APP-BP1 and Uba3, human Ubc12, NEDD8, Cul-1, and ROC1 were PCR-amplified and cloned into the pGEX or Duet vectors (Novagen) and pCDNA3.1 (Invitrogen). Cul-1 and ROC1 were co-expressed in *E. coli* by "Split-n-Coexpress" method [9]. APP-BP1 and Uba3 were co-expressed in *E. Coli* [10]. Their DNA sequences were verified (Applied Biosystems). All proteins were expressed in BL21 (DE3) RIL (Novagen) and purified using either Ni Sepharose 6 Fast Flow (GE Healthcare), TALON Affinity Resin (Clontech), size fractionation column and Glutathion Sepharose 4 Fast Flow (GE Healthcare). GST-tag was removed from GST-fusion proteins by anion exchange columns following cleavage by thrombin or prescission proteases (GE Healthcare).

In vitro NEDDylation assay. NEDDylation was performed on 30 °C in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM Dithiothreitol (DTT). The amounts of recombinant proteins used were as follows; APP-BP1/Uba3 200 ng, Ubc12 200 ng, Cul-1/ROC1 900 ng and NEDD8 250 ng. Methylated NEDD8 was purchased (Bostom Biochem).

For NEDD8 transfer assay, His-FLAG-Ubc12 charged with NEDD8 was immuno-purified by anti-FLAG M2 agarose (SIGMA) and FLAG-peptide (SIGMA), following pre-incubation with APP-BP1/Uba3 (3.6 μg), His-FLAG-Ubc12 (23 μg) and NEDD8 (2.5 μg) in the above reaction condition. His-FLAG-Ubc12 charged with NEDD8 was mixed with Cul-1/ROC1 or its derivatives.

Cell culture and transfections. HEK293 cells were grown in the medium supplemented with 10% fetal bovine serum and antibiotics. Expression plasmids for human NEDD8, Ubc12 and ROC1 and its mutant (C75A/H77A) were transfected by FuGENE6 (Roche).

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Cells were harvested after 48 h transfection in 1 ml of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 and subjected to the immunoprecipitation using Anti-FLAG-conjugating agarose beads (M2, SIGMA).

TPEN treatment. FLAG-Ubc12, HA-NEDD8 and 6Myc-ROC1 were co-transfected in HEK293 cells and TPEN (SIGMA) was added to cell cultures at indicated concentrations. After TPEN treatment, cells were lysed and immunoprecipitated using Anti-FLAG-conjugating agarose beads.

SDS–PAGE and Western blotting. All samples were separated by 10–15% SDS–PAGE and visualized by chemical luminescence (Chemiluminescence, Perkin-Elmer LAS, Inc.) following reaction with HRP-conjugated secondary antibodies. Anti-NEDD8, Anti-Uba3 and Anti-Ubc12 anti-sera were raised in Rabbit. Anti-FLAG (SIGMA), Anti-Cul-1 (ZYMED), Anti-HA (CONVANCE and Bethyl Laboratories), HRP-conjugated second antibodies (Jackson ImmunoResearch Laboratories) were purchased.

Results and discussion

Poly-NEDDyaltion of Cul-1 in vitro

It has been reported that Cul-1 can be hyper NEDDylated in vitro [11–13]. To reveal whether and how NEDD8 molecule forms poly-NEDD8 chain on Cul-1, we devised a complete in vitro reconstituted NEDD8 conjugation system composed of recombinant proteins purified from *E. coli* (i.e., APP-BP1, Uba3, Ubc12, NEDD8, Cul-1 and ROC1) (Fig. 1A). By mixing all proteins and incubation at 30 °C, NEDD8 conjugation to Cul-1 was observed (Fig. 1B). Omission of any component failed to conjugate NEDD8 to Cul-1 (Fig. 1B). These results suggest that all proteins were competent and could reproduce the NEDDylation of Cul-1 in vitro. While half of Cul-1 was mono-NEDDylated within 10 min incubation, a slower mobility shift of Cul-1 was observed at longer time of reaction such as 3 h (Fig. 1C). To confirm whether these slower mobility shift bands are due to poly-NEDDylation of Cul-1, we used methylated NEDD8, defective for chain formation. Higher molecular weight bands disappeared by using methylated NEDD8 (Fig. 1C). Furthermore, Cul-1(K720R) was not NEDDylated at all even at 3 h or longer incubation (Fig. 1D). These observations suggest that the slowly migrated bands are indeed due to poly-NEDDylation of Cul-1 at Lys-720 but not multiple mono-NEDDylation of Cul-1.

Poly-NEDD8 chain is formed on catalytic Cysteine residue of Ubc12

To further explore the molecular mechanism of poly-NEDD8 chain formation, we asked whether poly-NEDD8 chain could be formed on Ubc12, because it is known that ubiquitin can form poly-ubiquitin chain on ubiquitin E2 enzyme [14]. Western blotting of Ubc12 showed multiple Ubc12 bands with slower mobility shifts suggestive of poly- NEDDylation (Fig. 2A). These band shifts were not observed on Uba3 (Fig. 2A). Higher molecular weight bands did not appear by using methylated NEDD8, indicating that these bands correspond to poly-NEDDylated Ubc12 (Fig. 2B). However, some slowly migrated bands were still observed by methylated NEDD8, indicating that Ubc12 has multiple NEDDylation sites on its molecule. In the next step, we examined whether poly-NEDD8 chain is linked to the catalytic Cys-111 residue of Ubc12 via thioester bond. To verify this, DTT was added to poly-NEDDylated Ubc12 and blotted with Anti-NEDD8. Poly-NEDD8 chain was detected in DTT-treated, but not untreated sample indicating that some poly-NEDD8 chain is attached on the catalytic Cys-111 residue of Ubc12 via thioester bond (Fig. 2C). However, because not all of the poly-NEDD8 chain on Ubc12 was removed by DTT treatment, and Ubc12 still migrated to higher molecular weight, Ubc12 could be poly-NEDDylated at multiple NEDDylation site(s) (Fig. 2D).

ROC1 inactivation enhances poly-NEDD8 chain formation

To next elucidate the role of ROC1 for poly-NEDD8 chain formation, we devised a NEDD8 transfer assay by purifying His-FLAG-Ubc12 charged with activated NEDD8 (Fig. 3A). As shown in Fig. 3B, addition of Cul-1/ROC1 complex to this NEDD8 charged Ubc12 resulted in rapid transfer of NEDD8 from Ubc12 to Cul-1 in the absence of APP-BP1/Uba3, $MgCl_2$ and ATP. The NEDD8 transfer

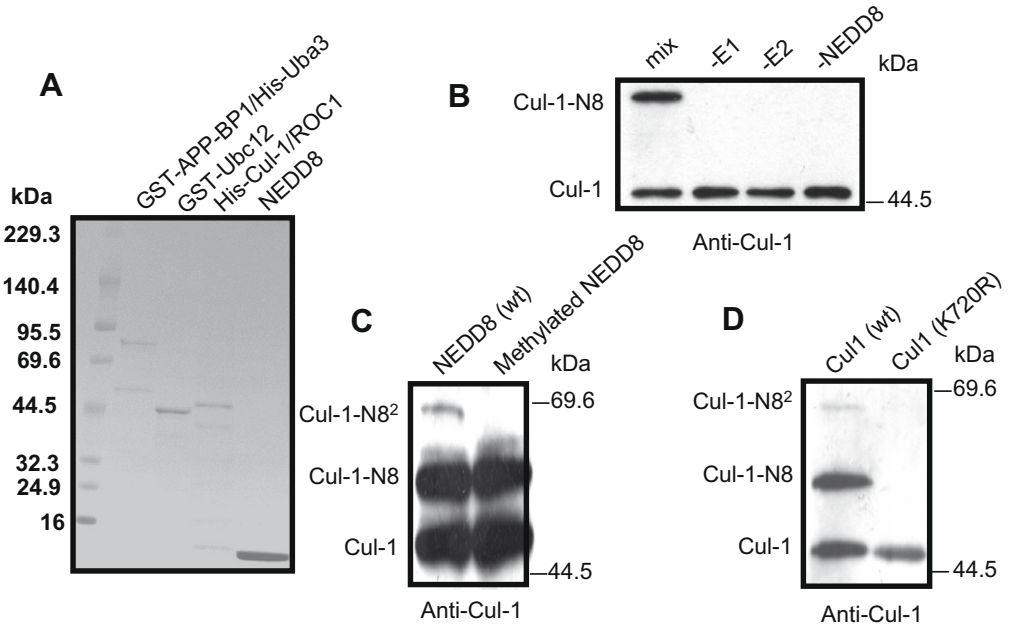

Fig. 1. In vitro NEDD8 reaction with recombinant proteins. (A) Coomassie staining of purified recombinant proteins. (B) Cul-1/ROC1 were mixed with E1 (GST-APP-BP1/His-Uba3), E2 (GST-Ubc12) and NEDD8. (C,D) In vitro NEDDylation reactions with NEDD8 or methylated NEDD8. Cul-1(K720R)/ROC1 was used as negative control in (D). Reaction products were blotted with Anti-Cul-1 antibody. Cul-1-N8 and Cul-1-$N8^2$ denote mono-NEDDylated and di-NEDDylated Cul-1, respectively.