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Assembly of different length of polyubiquitins on the catalytic cysteine of E2 enzymes without E3 ligase; a novel application of non-reduced/reduced 2-dimensional electrophoresis

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In this study using non-reduced/reduced 2-dimensional electrophoresis (NR/R-2DE), we clearly demonstrated that E3-independent ubiquitination by Ube2K produced not only unanchored but also Ube2K-linked polyubiquitins through thioester and isopeptide bonds. E3-independent assembly of polyubiquitins on the catalytic cysteine of Ube2K strongly supports the possibility of '*en bloc* transfer' for polyubiquitination. From the same analyses of E3-independent ubiquitination products by other E2s, we also found that different lengths of polyubiquitins were linked to different E2s through thioester bond; longer chains by Cdc34 like Ube2K, short chains by Ube2g2, and mono-ubiquitin by UbcH10. Our results suggest that E2s possess the different intrinsic catalytic activities for polyubiquitination.
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1. Introduction

Ubiquitin (Ub) is involved in almost every cellular function by regulating protein stability, interaction, activity, or localization through conjugation of many thousands of proteins [1–3]. In the conventional reaction, Ub is activated, transferred, and conjugated to a substrate through E1–E2–E3 cascade, during which Ub is sequentially transformed into adenylated Ub (Ub-AMP), thioester-linked Ub to enzyme (E∼Ub), and isopeptide-linked Ub to substrate (Ub-S). Each round of ubiquitination results in the formation of an isopeptide bond between C-terminus of Ub from E∼Ub and a specific lysine residue in a substrate recruited by an E3. Ub can be consecutively linked to one another on Ub-S through isopeptide bonds with repeated cycles of Ub transfer, yielding polyubiquitinated substrate (Ub_n-S). However, the detailed molecular mechanism of polyUb synthesis is largely unknown. Recently, '*en bloc* transfer' of preassembled polyUbs on E2 to a substrate was proposed as an alternative to one-by-one Ub transfer [4–6].

Although E3s recruit specific substrates, the length, topology, and processivity for polyubiquitination seem to be determined by the catalytic activities of E2s [7–9]. Recently, E2s are considered as major players in Ub transfer rather than simple Ub carriers [10]. In addition, it has been reported that not only unanchored but also enzyme-linked polyUbs can be synthesized by some E2s without any E3s [11–15]. In this study, we tried to analyze all possible products by in vitro E3-independent ubiquitination in order to investigate any differences in the catalytic activities of E2s.

Ube2K, also known as E2-25K or Hip2, is well known to catalyze the synthesis of unanchored polyUbs without E3s [11,16], implying that Ub-charged Ube2K (Ube2K∼Ub) can donate its activated Ub to the recruited free Ub or polyUb without the aid of an E3. In this study, we clearly demonstrated by a novel application of NR/R-2DE that Ube2K produced not only unanchored but also Ube2K-linked polyUbs through isopeptide and thioester bonds. E3-independent ubiquitination by other E2s produced similar polyUb products with the different lengths. Preassembly of polyUbs on catalytic cysteine of some E2s through thioester bond strongly supports the possibility of '*en bloc* transfer' mechanism in polyubiquitination.

2. Materials and methods

2.1. Materials

E1 was purchased from Boston Biochem (E-305, Cambridge, USA). Ub antibody was purchased from Santa Cruz Biotechnology (P4D1, Santa Cruz, USA). pET21a vector was from Novagen (Darmstadt,

Abbreviations: NR/R-2DE, non-reduced/reduced 2-dimensional electrophoresis; Ub, ubiquitin; ∼Ub, thioester-linked Ub; Ub-, isopeptide-linked Ub

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Germany). Pre-casting gels were from KOMA Biotech (Seoul, South Korea).

2.2. Preparation of proteins

Synthetic Ub gene with *Escherichia coli* optimized codons was cloned into pET21a vector to generate expression vector of Ub (pET21a/Ub) as described previously [17]. Expression vectors for Ub^{K0} and $Ub^{\Delta GG}$ were generated from pET21a/Ub by site-directed mutagenesis. Ub and its derivatives were expressed in the transformed *E. coli* BL21(DE3) by IPTG induction and purified by size-exclusion chromatography after heat-treatment.

E2 genes obtained by PCR of cDNAs encoding Ube2K (NM_005339.4), Ube2g2 (NM_003343.5), Cdc34 (NM_004359.1), and UbcH10 (NM_007019.2) were cloned into pET21a expression vector with N-terminal histidine tag. Ube2K(C92S) and Ube2K(K97R), were prepared by site-directed mutagenesis. All histidine-tagged proteins were expressed in BL21(DE3) *E. coli* strain and purified by using Ni-NTA column followed by desalting with buffer (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 8.1 mM KH_2PO_4. Protein concentration was determined by the BCA method.

2.3. In vitro ubiquitination assay

E2 (1 μM) were incubated with 0.1 μM E1, 10 μM Ub, and 4 mM ATP at 37 °C in the buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, and 0.1 mM DTT. Samples were quenched by SDS-sample buffer without reducing reagent. For reduced condition, samples were treated with 5% β-mercaptoethanol before SDS–PAGE. DiUb synthesis assays were carried out similarly, except that 5 μM Ub^{K0} and 5 μM $Ub^{\Delta GG}$ were used instead of 10 μM Ub.

2.4. SDS–PAGE and immunoblotting

All samples were not boiled and ubiquitination products in reduced or non-reduced condition were separated by 4–12% SDS–PAGE under the Tris–aspartate running buffer (pH 6.0) containing 100 mM Tris-base, 100 mM aspartic acid, and 0.1% SDS. Tris–glycine running buffer containing 25 mM Tris-base, 190 mM glycine, and 0.1% SDS was used for the better resolution of high molecular weight products. Proteins in gels were transferred onto nitrocellulose membranes under 12 mM Tris, 96 mM glycine, and 20% methanol. Ubiquitination products from the Western blot were visualized with ECL reagent (GE LifeScience, USA).

2.5. Non-reduced/reduced 2-dimensional electrophoresis (NR/R-2DE)

After the separation of ubiquitination products under non-reduced condition by 10% SDS–PAGE, each gel lane was cut out, incubated with 65 mM DTT for 15 min, loaded horizontally onto a stacking gel, and covered with sample buffer. Then, proteins were separated perpendicularly during the second electrophoresis under reduced conditions. Ubiquitination products were also separated by reduced/reduced two-dimensional electrophoresis (R/R-2DE) for the control.

3. Results

3.1. E3-independent synthesis of Ub chains by Ube2K

All products from in vitro E3-independent ubiquitination by Ube2K were analyzed by anti-Ub immunoblotting (Fig. 1A). Overall ubiquitination products were increased with reaction time, among which three different groups could be discriminated; (I) unanchored Ub chains that gradually increase its chain length with time (Ub_2, Ub_3, and Ub_4), (II) majority of products as self-ubiquitinated Ube2K (Ub_n–Ube2K), and (III) high molecular weight ubiquitinated products (Fig. 1A).

Generation of all the ubiquitinated products was absolutely dependent on the active site cysteine (C92) of Ube2K (Fig. 1B). When the 97th lysine residue (K97) was replaced with an arginine residue, the ubiquitination products were significantly reduced mainly in group II, indicating that isopeptide-linked Ub chains to Ube2K (Ub_n–Ube2K) were the major ubiquitination products in this group (Fig. 1B). With better resolution of high molecular weight products, smear bands in group III could be separated into discrete ladder-type bands under the reduced condition (R lane in Fig. 1C), which represented unanchored (Ub_n) and isopeptide-linked Ub chains to enzymes (Ub_n–E1 or Ub_n–Ube2K). Interestingly enough, significantly more ubiquitinated products were detected under the non-reduced condition (NR lane in Fig. 1C), indicating that various length of Ub chains were linked to active site cysteine of enzymes through thioester bonds such as E1∼Ub_n and Ube2K∼Ub_n. These results clearly demonstrated that in vitro E3-independent ubiquitination by Ube2K simultaneously produced three different forms of Ub chains such as unanchored (Ub_n), isopeptde-linked (Ub_n-), and thioester-linked ones (∼Ub_n).

3.2. Ub chains are assembled on the active site cysteine of Ube2K through thioester bond

In this study, we applied NR/R-2DE in order to separate ∼Ub_n among E3-independent ubiquitination products. During NR/R-2DE, thioester bonds are maintained under non-reduced condition in the first dimensional electrophoresis, but easily cleaved under the reduced condition in the second dimensional electrophoresis, so that ∼Ub_n are released from enzymes, migrate separately, and can be detected as new spots by anti-Ub immunoblotting.

In an immunoblot after R/R-2DE as a control, all Ube2K-mediated ubiquitination products in group I, II, and III were detected as spots in one major diagonal line (Fig. 2A). In contrast, additional diagonal line of Ub chains was detected below a major one in an immunoblot after NR/R-2DE, in which Ub_n and Ub_n- are positioned in the major diagonal line whereas ∼Ub_n in a new diagonal line after releasing from enzymes during the second dimensional electrophoresis (Fig. 2B). These results indicated that not only monoUb but also various length of Ub chains were linked to the active site cysteine of Ube2K through thioester bond. Since two diagonal lines were convergent in high molecular weight region in Fig. 2B, we analyzed the ubiquitination products in group III by a separate NR/R-2DE, and resolved ∼Ub_n from Ub_n and Ub_n- (Fig. 2C). These results clearly demonstrated that various length of Ub chains were linked to Ube2K through thioester bond but not to E1 (Fig. 2C). The numbers of Ubs in Ube2K∼Ub_n reached to more than two dozen based on molecular weight of Ub chains detected on a second diagonal line (Fig. 2C).

3.3. Preassembly of Ub chains on the active site cysteine is an intrinsic property of Cdc34 and Ube2g2, but not UbcH10

Since we found that Ube2K could simultaneously catalyze the synthesis of various ubiquitination products in vitro without any E3s, we examined the E2-mediated ubiquitination with Cdc34 and Ube2g2, which are known to catalyze K48-linked Ub chains like Ube2K [5,18]. When Cdc34-mediated ubiquitination products were separated by 1D-PAGE under reduced and non-reduced conditions and visualized by anti-Ub immunoblotting, high molecular weight products were detected significantly more in non-reduced condition compared with those in reduced condition, indicating that various polyUb chains were linked to active site cysteine of

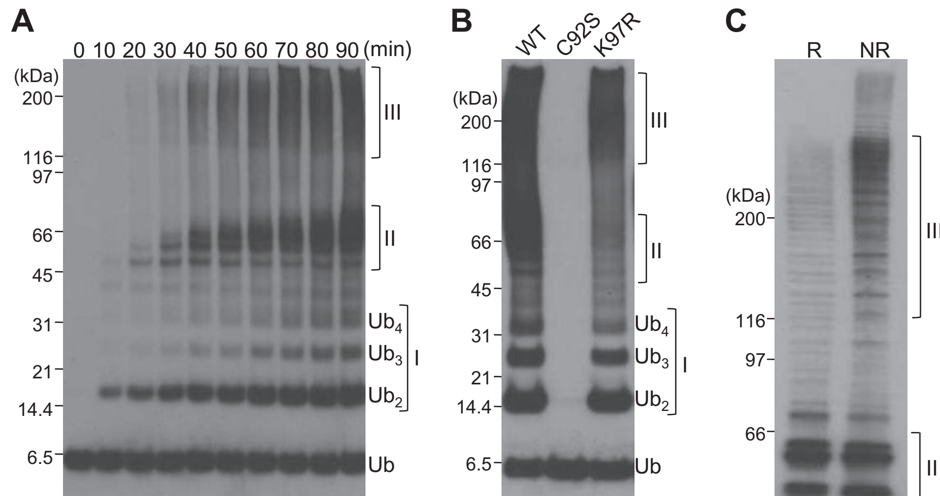

Fig. 1. E3-independent ubiquitination by Ube2K in vitro. Ube2K (1 μM) was incubated with 0.1 μM E1, 10 μM Ub, and 4 mM ATP for 90 min and ubiquitination products were analyzed by anti-Ub immunoblotting after SDS–PAGE. (A) Overall ubiquitination products gradually increased with time, among which three different groups could be discriminated; (I) unanchored Ub chains (Ub_2, Ub_3 and Ub_4), (II) self-ubiquitinated Ube2Ks, and (III) high molecular weight ubiquitination products. (B) Ube2K(C92S) could not synthesize any ubiquitination products whereas Ube2K(K97R) produced significantly less products in group II. (C) With better resolution of high molecular weight products, discrete ladder-type bands were detected under reduced condition (R), and significantly more ubiquitinated products were detected in non-reduced condition (NR).

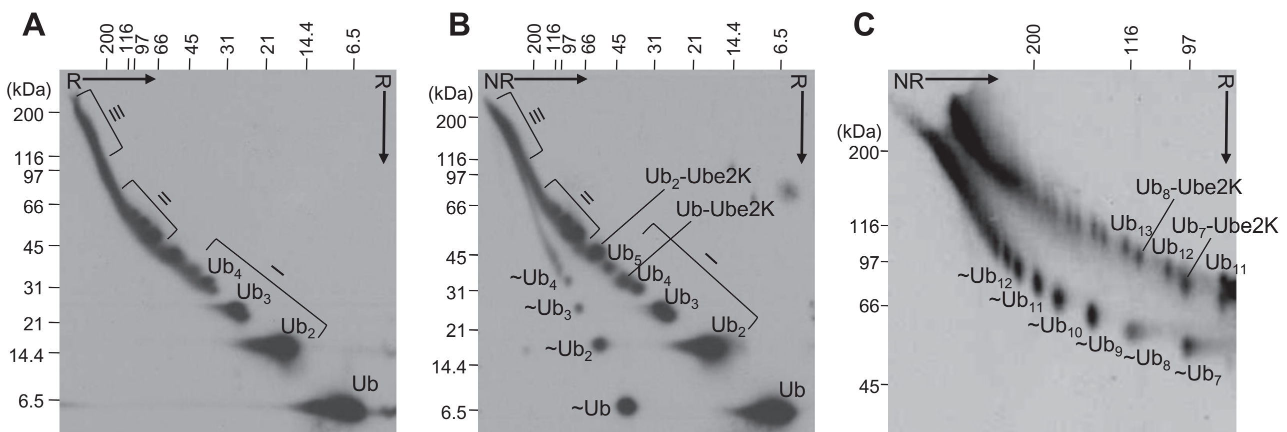

Fig. 2. Thioester-linked Ub chains to catalytic cysteine of Ube2K. Ubiquitination products from 1 h incubation with 0.1 μM E1, 1 μM Ube2K, 10 μM Ub and 4 mM ATP at 37 °C were analyzed by anti-Ub immunoblotting after 2DE. (A) All ubiquitination products in group I, II, and III were detected as spots in a diagonal line in an immunoblot after R/R-2DE. (B) Thioester-linked Ub chains were released from Ube2K$\sim$$Ub_n$ and detected as spots ($\sim$$Ub_n$) in a new diagonal line in an immunoblot after NR/R-2DE. Unanchored Ub chains (Ub_n) and isopeptide-linked Ub chains to Ube2K (Ub_n-Ube2K) were positioned in a major diagonal line. (C) With the better resolution of high molecular weight products in group III, $\sim$$Ub_n$ released from Ube2K$\sim$$Ub_n$ could be discriminated from Ub_n and Ub_n-Ube2K.

Cdc34 similar to Ube2K (Fig. 3A). Assembly of polyUb chains on Cdc34 through thioester bonds was further confirmed by a second diagonal line of spots in an anti-Ub immunoblot after NR/R-2DE compared with one after R/R-2DE (Fig. 3B and C). Overall pattern of Cdc34-mediated ubiquitination products were similar to those by Ube2K. It is noticeable that Ub released from the Ub-charged E1 (E1$\sim$Ub) was clearly detected in this immunoblot (arrow in Fig. 3C).

E3-independent ubiquitination products by Ube2g2 also produced Ub_n, Ub_n–Ube2g2, and Ube2g2$\sim$$Ub_n$, but with the limited number of Ub chains (Fig. 3D–F). Unlike the ubiquitination products by Ube2K and Cdc34, at least three monoUb spots (Fig. 3F) were detected in an anti-Ub immunoblot after NR/R-2DE of Ube2g2-mediated ubiquitination products, which were presumably originated from Ube2g2$\sim$Ub, Ub_1–Ube2g2$\sim$Ub, and Ub_2–Ube2g2$\sim$Ub, respectively. Corresponding spots for Ub_1–Ube2g2, and Ub_2–Ube2g2 after releasing $\sim$Ub were also detected in the same immunoblot (arrowheads in Fig. 3F). Similarly, two Ub_2 spots in Fig. 3F might be originated from Ube2g2$\sim$$Ub_2$ and Ub–Ube2g2$\sim$$Ub_2$. Our results indicate that Ub_n–Ube2g2 can be linked with Ub chains through thioester bonds, generating the isopeptide/thioester-linked Ub chains to Ube2g2 (Ub_n–Ube2g2$\sim$$Ub_n$).

We also analyzed the E3-independent products by UbcH10, which was known to involve mainly in mono-ubiquitination [19,20]. It was found that the catalysis by UbcH10 preferred the isopeptide-linked Ub chains to the unanchored ones (Fig. 3G). In an immunoblot after NR/R-2DE, only monoUb was detected as a new spot (Fig. 3H and I), indicating that UbcH10 was unable to assemble Ub chains on its active site via a thioester bond unlike previous E2s. Appearance of Ub–UbcH10 as a new spot (arrowhead in Fig. 3I) implied that Ub–UbcH10 could be potentially charged with the activated donor Ub like Ube2g2, although the released monoUb from Ub–UbcH10$\sim$Ub was barely detected (asterisk in Fig. 3I).

In this study, we tried to analyze all possible E3-independent ubiquitination products by four different E2s, but did not directly compare their catalytic activities. Although various forms of Ub chains were produced, the ultimate catalytic activity of E2 is the

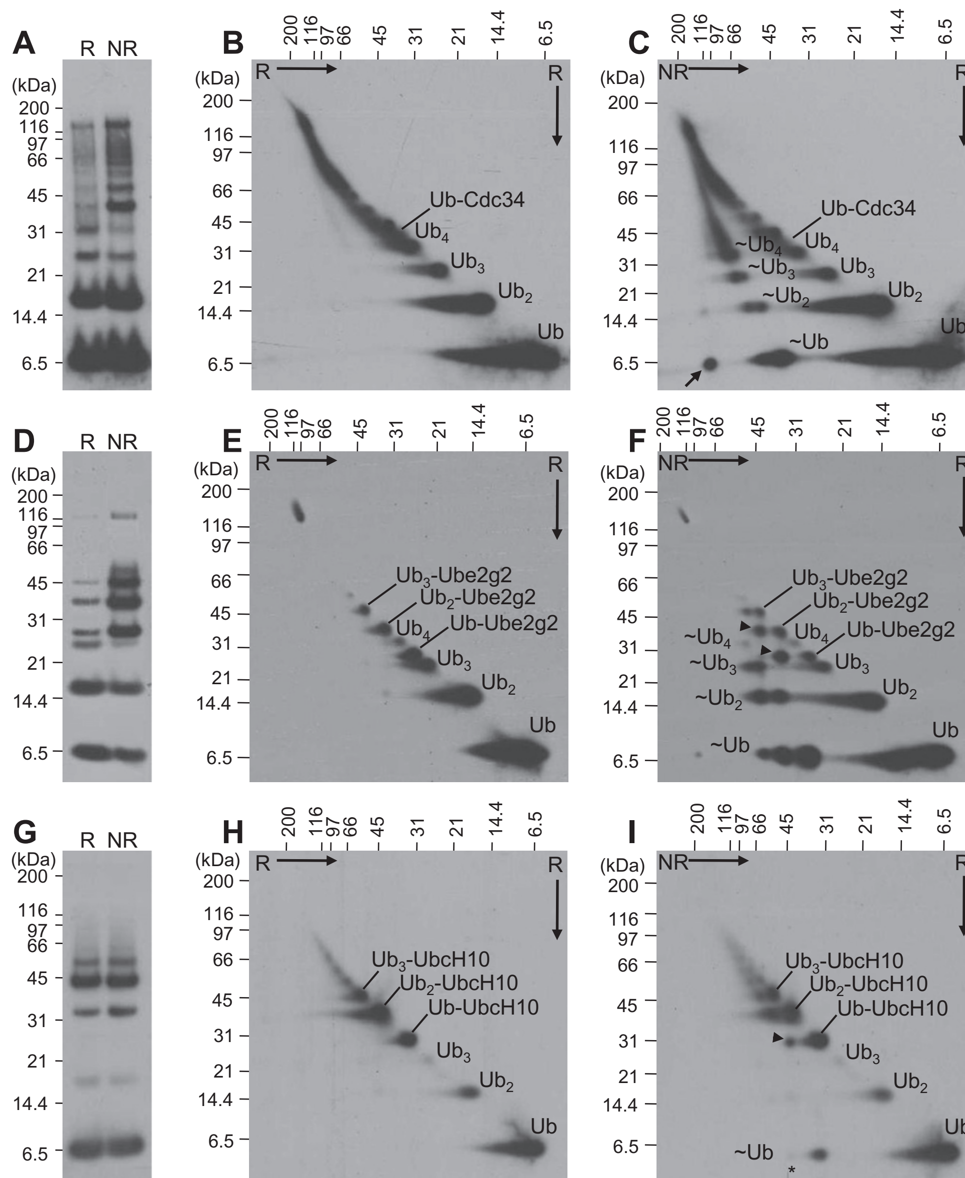

Fig. 3. E3-independent synthesis of Ub chains by Cdc34, Ube2g2, and UbcH10. E3-independent ubiquitination products by Cdc34, Ube2g2, and UbcH10 were analyzed by anti-Ub immunoblotting after SDS–PAGE (A, D, and G), R/R-2DE (B, E, and H) and NR/R-2DE (C, F, and I). The spots of the unanchored Ubs, isopeptide-linked Ubs to enzyme, and the released Ubs from thioester-linked Ubs were labeled as Ub_n, Ub_n–(E), and $\sim Ub_n$, respectively. R, reduced condition; NR, non-reduced condition

formation of isopeptide bonds. Therefore, we directly compared the catalytic rates among four E2s by a simple diUb synthesis assay using Ub^{K0} and $Ub^{\Delta GG}$ as donor Ub and acceptor Ub, respectively. It was found that the catalytic rates for isopeptide bond formation were different among those E2s; the most active by Ube2K, moderate by Cdc34 and Ube2g2, and the least by UbcH10 (Fig. 4).

4. Discussion

Accumulating evidences support that some E2s catalyze the synthesis of various forms of polyUbs with E1 in the absence of any E3s [10–15]. In this study, we applied the NR/R-2DE to separate the thioester-linked Ubs to enzymes from other products, and clearly demonstrated that E3-independent ubiquitination by Ube2K simultaneously produced three different forms of polyUbs such as unanchored (Ub_n), isopeptide-linked (Ub_n–Ube2K), and thioester-linked ones (Ube2K$\sim Ub_n$). At a catalytic level, ubiquitination for the synthesis of polyUbs ultimately occurs by the nucleophilic attack of the lysine residue of acceptor Ub on the thioester bond linking the E2 catalytic cysteine to donor Ub [2,4]. Therefore, E3-independent production of Ube2K$\sim Ub_n$ implies that Ub_n on Ube2K$\sim Ub_n$ can be the donor Ub or the acceptor Ub, resulting in the preassembly of polyUb on its active site cysteine. However, such products are difficult to detect in vivo, presumably due to the low abundance, the labile nature of the thioester bonds, and generally reducing conditions in cells [6,21].

Cdc34 and Ube2g2 could also catalyze the synthesis of thioester-linked Ub chains on its catalytic cysteine, producing Cdc34$\sim Ub_n$ and Ube2g2$\sim Ub_n$, respectively, but with different number of Ub extensions. In addition, only monoUb was linked to UbcH10 through thioester bond. Therefore, E2s seem to possess the different ability to extend the Ub chains. In addition, the catalytic rates for the formation of isopeptide bonds were also different among those E2s (Fig. 4).

We also found that some E2s such as Ube2K and Cdc34 cannot be linked with Ub chains through thioester bonds once they are linked

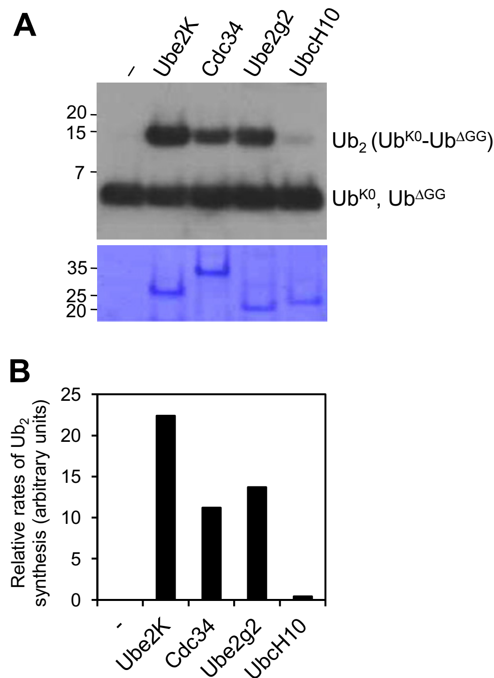

Fig. 4. Comparison of catalytic activities among E2s by a diUb synthesis assay. Ube2K, Cdc34, Ube2g2 or UbcH10 (1 μM each) was incubated with 0.1 μM E1, 5 μM Ub^{K0}, 5 μM $Ub^{\Delta GG}$ and 4 mM ATP at 37 °C for 1 h. The products of Ub_2 (Ub^{K0}–$Ub^{\Delta GG}$) were visualized by anti-Ub immunoblotting after SDS–PAGE (upper panel in A), which were quantitatively compared by densitometer (B). Purified recombinant E2s used in this assay were stained with Coomassie brilliant blue (lower panel in A).

with Ub chains through isopeptide bonds (Figs. 2C and 3C), suggesting that the self-ubiquitinated E2s cannot be charged with activated Ubs. However, other E2s such as Ube2g2 and UbcH10 can be linked the Ub chains through both isopeptide and thioester bonds to the same molecule, producing Ub_n–Ube2g2∼Ub_n and Ub_n–UbcH10∼Ub (Fig. 3F and I), which suggests that the self-ubiquitinated E2s can be still activated.

In this study, we clearly demonstrated that some E2s, without the help of a cognate E3, can catalyze the synthesis of different forms of Ub chains including unanchored (Ub_n), isopeptide-linked (Ub_n–E2), thioester-linked (E2∼Ub_n), or/and isopeptide/thioester-linked ones (Ub_n–E2∼Ub_n). E3-independent preassembly of Ub chains on the catalytic cysteine of E2s strongly supports the possibility of '*en bloc* transfer' for polyubiquitination. Taken together, our results demonstrated that each E2 possesses the different catalytic activities for the ubiquitination in aspect of the ability of Ub extension, the rates of peptide bond formation, and the preference for the synthesis among different Ub chains, which may be promoted or modulated by an E3 in vivo.

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