Differential Ubiquitin Binding by the Acidic Loops of Ube2g1 and Ube2r1 Enzymes Distinguishes Their Lys-48-ubiquitylation Activities*

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Background: The acidic loops of E2 enzymes are important for their Lys-48-ubiquitylation activity.
Results: The presence of Tyr residues modulates the ubiquitin binding property of the acidic loop.
Conclusion: A proper interaction of the acidic loop with the attached donor ubiquitin is important for Lys-48-ubiquitylation activity.
Significance: One of molecular bases to study the mechanism of Lys-48-ubiquitylation is provided.

The ubiquitin E2 enzymes, Ube2g1 and Ube2r1, are able to synthesize Lys-48-linked polyubiquitins without an E3 ligase but how that is accomplished has been unclear. Although both E2s contain essential acidic loops, only Ube2r1 requires an additional C-terminal extension (184–196) for efficient Lys-48-ubiquitylation activity. The presence of Tyr-102 and Tyr-104 in the Ube2g1 acidic loop enhanced both ubiquitin binding and Lys-48-ubiquitylation and distinguished Ube2g1 from the otherwise similar truncated Ube2r1 (1–183) (Ube2r1C). Replacement of Gln-105−Ser-106−Gly-107 in the acidic loop of Ube2r1C (Ube2r1CYGY) by the corresponding residues from Ube2g1 (Tyr-102−Gly-103−Tyr-104) increased Lys-48-ubiquitylation activity and ubiquitin binding. Two E2−UB thioester mimics (oxyster and disulfide) were prepared to characterize the ubiquitin binding activity of the acidic loop. The oxyster but not the disulfide derivative was found to be a functional equivalent of the E2−UB thioester. The ubiquitin moeity of the Ube2r1CYGY-[15N]UBK48R oxyster displayed two-state conformational exchange, whereas the Ube2r1CYGY-[15N]UBK48R oxyster showed predominantly one state. Together with NMR studies that compared UBK48R oxysters of the wild-type and the acidic loop mutant (Y102G/Y104G) forms of Ube2g1, in vitro ubiquitylation assays with various mutation forms of the E2s revealed how the intramolecular interaction between the acidic loop and the attached donor ubiquitin regulates Lys-48-ubiquitylation activity.

The modification of proteins by ubiquitylation is important for the regulation of a large variety of cellular processes that includes cell cycle control, endocytosis, DNA repair, and regulation of transcription (1, 2). E3 ligases mediate the formation of isopeptide bonds between the C-terminal carboxylic group of ubiquitin and a Lys side chain of the target protein (monoubiquitylation) or a previously attached ubiquitin (polyubiquitylation). Although many kinds of ubiquitin-ubiquitin linkages are present in vivo (3), Lys-48-linked polyubiquitin chains, which direct ubiquitylated proteins to the 26 S proteasome complex for their degradation, are the most abundant ubiquitin signals. RING-E3 ligases are the dominant E3 species that mediate the direct transfer of ubiquitin (4) from the E2−UB thioester intermediate to a target protein (2). An interaction between E2−UB and ubiquitin is likely to be necessary for efficient Lys-48 polyubiquitylation, but such an interaction remains poorly characterized. In contrast, for Lys-63-linked ubiquitylation by the UbE13 (E2) and Mms2 (ubiquitin-E2 variant) complex, Mms2 recruits an acceptor ubiquitin and positions its Lys-63 side chain in close proximity to the active site Cys of UbC13 (5, 6). In a recent molecular docking model for Lys-11-ubiquitin linkage formation by Ube2S (7) based on NMR chemical shift perturbation (CSP) data and in vitro ubiquitylation assay, Ube2S has binding surfaces for both acceptor and donor ubiquitin to position them and generate Lys-11-linkage polyubiquitin, whereas Ubc13 needs Mms2 to bind to and position an acceptor ubiquitin. In this model the interaction between donor ubiquitin or acceptor ubiquitin and Ube2S was very weak. Analogous mechanisms for the formation of Lys-48 ubiquitin linkages are unknown, possibly because interactions that guide Lys-48-ubiquitylation may be very weak and difficult to identify.

An in vitro E3-independent ubiquitylation assay that used p27−E2 fusion proteins had identified Ube2d2 (UbcH5b) and

*This work was supported, in whole or in part, by National Institutes of Health Grant GM097452 (to R. E. C.). This study was also supported by the high-field NMR research program from Korea Basic Science Institute (KBSI; to K.-S. R.) and KBSI Grant T33410 (to H.-K. C.).
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Ube2b (hHR6b) as the most efficient mediators for the ubiquitylation of p27, but those E2s were found to be less effective for subsequent Lys-48-ubiquitylation in comparison to Ube2r1 (human Cdc34) (8). Recent reports showed that the sequential engagement of two different E2 enzymes may be important for efficient polyubiquitylation of target proteins in an E3-dependent system. UbcH5 was found to be efficient for the first ubiquitylation of IkBa, but then Ube2r1 performed the subsequent Lys-48-ubiquitylation reactions in the presence of Skip/Cullin/F-box E3 complex (9). Ube2r1 is able to synthetize Lys-48-linked polyubiquitin chains efficiently without an E3 ligase. This E3-independent reaction mechanism is likely to be used as well by the E2 during the RING-E3-mediated ubiquitylation.

Both Ube2g1 (human Ubc7) and Ube2r1 possess an acidic loop in the vicinity of the active-site Cys residue and display an exceptional ability to synthesize Lys-48-linked polyubiquitin chains. The acidic loops contain several Asp and Glu residues, and mutations eliminating these negative charges have been shown to decrease the ubiquitylation activity of yeast Cdc34 in the presence of the Skip/Cullin/F-box E3 complex (10). Ube2k, which lacks the acidic loop, has a similar ability for Lys-48-specific polyubiquitylation, whereas most other E2 enzymes cannot synthesize Lys-48 polyubiquitin efficiently in the absence of an E3 ligase (11). Ube2g1 consists of only an E2-core domain, but Ube2r1 has an additional C-terminal extension (residues 184 – 236). Work from our group previously showed that, unlike full-length Ube2r1, the Ube2r1 core (residues 1 – 183; Ube2r1C) has a decreased capability to synthesize Lys-48 polyubiquitin chains efficiently in the absence of an E3 (8). Ube2d2, which does not have an acidic loop, also is unable to synthesize Lys-48 polyubiquitin in the absence of an E3 ligase (8) despite having ubiquitin binding affinity almost identical to that of Ube2g1 as assessed by NMR CSP experiments using [15N]UB (12).

In this study we focused on the acidic loops and ubiquitin binding activities of Ube2g1 and Ube2r1C. By comparing ubiquitin binding and polyubiquitin synthesis by Ube2g1, Ube2r1C, and Ube2d2, their different abilities to synthesize Lys-48-linked chains were evaluated with respect to intramolecular interaction between the acidic loop and the attached donor ubiquitin.

**Experimental Procedures**

**Protein Preparations**—Genes encoding E2 enzymes (ube2g1, ube2r1, and ube2d2) and ubiquitin variants (His- or non-tagged) were cloned into the pGEX-4T-1 (BamHI/XhoI) and pET15b/Harbor Digestion following by thrombin inactivation with 1 mM PMSF and benzamidine-HCl. Target proteins were further purified by gel permeation chromatography using Superdex-75 (GE Healthcare); where applicable, subsequent passage through a GSTrap column was used to remove the cleaved GST. Murine E1 protein was purified following a previously reported method (8), and the concentration of the E1 stock solution (~2.0 mg/ml) was estimated based on band intensity observed after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie Blue.

All of the isotope-enriched proteins for NMR experiments were obtained by growing E. coli in M9 minimal medium supplemented with [15N]NH4Cl (0.1%) and [13C]glucose (0.2%). The (i) random fractional (75 – 80%) and (ii) complete (~97%) deuteration of proteins were done by growing E. coli in M9 minimal medium (99% D2O) supplemented with (i) [13C]glucose (0.2%) and [15N]NH4Cl (0.1%) or (ii) [13C,2H]glucose (0.2%), 13C,15N-labeled Celton powder (0.1%, Cambridge Isotope Laboratory), and [15N]NH4Cl (0.1%), respectively (13, 14).

**In Vitro Ubiquitylation Assay—In vitro ubiquitylation was done using 200 – 300 μM ubiquitin, 20 μM E2, and ~0.5 μM E1 in a reaction buffer (pH 7.0, 7.5, or 8.0) composed of 50 mM Tris-HCl, 1 mM DTT, 2 mM ATP, 2 mM MgCl2, 10 mM creatine phosphate, and 1 unit each of pyrophosphatase and creatine phosphokinase at 25 °C. The reaction products were separated by 14 – 16% SDS-PAGE. The formation of E2-UB thioester was evaluated by non-reducing SDS-PAGE in which the reaction mixtures were sampled 20 min after the addition of E1 and then quickly frozen in liquid nitrogen.

To estimate the approximate $K_m$ values for E2-mediated Lys-48-ubiquitylation, the in vitro ubiquitylation assays were performed with 1.5 μM E1 and increasing ubiquitin concentrations from 0.2 to 2.4 mM. The amounts of diubiquitin synthesized were estimated by integrating the diubiquitin bands in the Coomassie Blue-stained SDS-PAGE gel using the Multi Gauge program (Fujifilm). The reaction sample containing the highest concentration of the ubiquitin substrate was serially diluted and separated in the same SDS-PAGE as an internal reference to confirm the linearity of the integrated band intensities. Sample loadings were reduced to ensure that the volume integrals of diubiquitin bands correlated linearly to the amounts loaded; two or more experimental replicates were done for each E2 enzyme. The establishment of a stable concentration of E2-ubiquitin thioester was associated with a time lag during which the product was not synthesized constantly. Therefore, the amount of synthesized di-UB is lower than expected, and this error was high at low concentrations of substrate ubiquitin. Nevertheless, the volume integrals of the diubiquitin bands were fitted with the Michaelis-Menten equation to estimate relative $K_m$ values of different E2s.

**Preparation of E2-UB Oxyster and E2-UB Disulfide Derivatives**—E2-UB oxyster was synthesized using the active-site Cys-to-Ser mutant E2 protein (Ube2g1C90S or Ube2r1C93S) in reactions with a UB$^{K48R}$, E2 molar ratio of 2. Instead of wild-type UB, intact or His$_6$-tagged UB$^{K48R}$ was used here to prevent loss of E2-UB oxyster caused by Lys-48 diubiquitin formation and to facilitate product purification. The amounts of E1 used were up to 10× larger than was used in the standard ubiquitylation assay; more E1 was required for Ube2g1C90S than for Ube2r1C93S due to the higher hydrolysis rate of Ube2g1C90S-UB$^{K48R}$ oxyster. The ubiquitin-coupling reactions were done in a buffer (pH 8.0) consisting of 50 mM Tris-HCl, 1 mM DTT, 4 mM ATP, 5 mM MgCl$_2$, 20 mM creatine phosphate, and 2 units of pyrophosphatase and creatine phosphokinase. The reaction

2252 JOURNAL OF BIOLOGICAL CHEMISTRY

ASBMB

VOLUME 290 • NUMBER 4 • JANUARY 23, 2015
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**RESULTS**

Despite Their Similar Domain Structures, the Lys-48 Polyubiquitylation Activity of Ube2r1C Is Much Lower Than That of Ube2g1—Sequence alignment of several E2 enzymes (Ube2g1, Ube2g2, Ube2r1, yeast Cdc34, Ube2d2, and Ube2k) shows that Ubc7 homologs (Ube2g1 and Ube2g2) and Cdc34 homologs (Ube2r1 and yeast Cdc34) have a distinct 12- or 13-residue insertion sequence in the vicinity of the active-site Cys (Fig. 1); this sequence was designated as an acidic loop due to the pres-

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**FIGURE 1. Structure-based sequence alignment of E2 enzymes.** The sequence alignment was performed through structural matching of the E2 enzymes using the Chimera program (24). The previously identified non-covalent ubiquitin-binding sites of the Ube2r1 C-terminal tail (26) are marked with a gray highlight, and the acidic loop region is indicated by a yellow highlight. The asterisk represents the active-site Cys residues of the E2 enzymes. Ube2g1 and Ube2r1 residues that when mutated resulted in a decrease in Lys-48-ubiquitylation activity are indicated by open arrows, those that have no activity change are indicated by closed arrows. The surface of Ube2g1 is roughly divided into four areas for better description of the data; the bottom side (red bar), the acidic loop (green bar), the left side (blue bar), and the right side (gray bar).
ence of many negatively charged Asp and Glu residues in yeast Cdc34 (10) (Fig. 1). Both Ube2g1 and Ube2r1 can synthesize Lys-48-linked polyubiquitin adducts in the absence of E3 ligase. However, despite the close similarity of their sequences (52% identity and 68% similarity), the 
in vitro ubiquitylation activity of Ube2r1 core enzyme (Ube2r1C, residues 1–183) was much lower than that of either Ube2r1 or Ube2g1. 
8, the Ube2r1 mutant proteins (Y190G and Y190G/V192G) showed significant impairment in their 
in vitro ubiquitylation activities. The insertion of a GGSGG sequence between Val-183 and Pro-184 of Ube2r1 resulted in a complete loss of Lys-48 ubiquitylation activity. C, Ube2r1C 
GGY had Gin-105–Ser-106–Gly-107 in the acidic loop replaced by the Tyr-102–Gly-103–Tyr-104 sequence from Ube2g1. Although the Lys-48-ubiquitylation activity of Ube2r1C was lower than that of Ube2g1, Ube2r1C 
GGY showed activity to a level close to that of Ube2g1 (left panel). The formation of E2–UB thioester adducts by Ube2g1, Ube2r1C, and Ube2r1C 
GGY was assessed by non-reducing SDS-PAGE analysis. Samples were prepared in SDS-PAGE loading buffer without β-mercaptoethanol 15 min after the ubiquitylation reaction was initiated with ATP. The three enzymes showed similar amounts of E2–UB thioester intermediate formation (right panel).

Ubiquitylation assays in which the E2 concentration was varied showed that the length of Lys-48 polyubiquitin products increased with increasing the concentration of Ube2r1 (Fig. 3). This result is consistent with the previous report that the dimerization of Ube2r1 increases its Lys-48-ubiquitylation activity (25). On the other hand, with Ube2g1 the size distribution of Lys-48 polyubiquitin products did not depend on the E2 concentration, and thus catalysis by Ube2g1 likely involves a reaction between the E2–UB thioester and an acceptor ubiquitin that is not associated with a second E2–UB molecule. In comparison with Ube2r1, the relatively simple ubiquitylation reaction by Ube2g1 suggested that studies focused on Ube2g1 and comparisons with Ube2d2 and Ube2r1C might reveal a mechanistic relationship between the ubiquitin binding activity and Lys-48-ubiquitylation.

Two Aromatic Residues (Tyr-102 and Tyr-104) in the Ube2g1 Acidic Loop Are Important for Non-covalent Ubiquitin Binding—

Previous NMR CSP experiments using [15N]UB showed that the ubiquitin binding properties of Ube2d2 are similar to those of
Ube2g1 (12), although Ube2d2 lacks an acidic loop and also cannot synthesize Lys-48 polyubiquitin chains efficiently in the absence of an E3 (8). We now have compared the ubiquitin-interacting surfaces of Ube2g1 and Ube2d2 in detail. Interestingly, CSP signals from the acidic loop region of Ube2g1 (Loop) were clearly identified when ubiquitin was added (Fig. 4, A and B, top panel). Two other major non-covalent ubiquitin binding surfaces of Ube2g1 (bottom and L-side) are very similar to those in Ube2d2 (data not shown). The presence of three ubiquitin binding surfaces in Ube2g1 (Kd/H11005 1.25 ± 0.39, 1.32 ± 0.40, and 1.46 ± 0.36 mM for bottom, L-side, and loop regions, respectively) can explain the lower apparent Kd value (0.56 ± 0.17 mM) that was previously determined from the CSP experiments using [15N]UB and Ube2g1 (12). The acidic loop in Ube2g1 is similar to the previously characterized weak ubiquitin-binding site 1 (UBS1) in the C-terminal tail of Ube2r1 (DLFYDDYYED) (26), which also consists of hydrophobic and acidic residues (Fig. 1). The T1, T2, and 1H,15N heteronuclear NOE values measured for Ube2g1 showed that the acidic loop region is more flexible than the bulk of the structured E2 core (data not shown); this finding is consistent with recent NMR 15N relaxation data for Ube2g2 (27).

Sequence alignment of Ube2g1 and Ube2r1C revealed two critical differences in their acidic loop regions: Tyr-102 and Tyr-104 in Ube2g1 correspond to Gln-105 and Gly-107 in Ube2r1 (Fig. 1). To assess the effect of Tyr-102 and Tyr-104 on non-covalent ubiquitin binding by Ube2g1, both were mutated to Gly residues (Ube2g1Y102G/Y104G). After the assignment of the backbone chemical shifts of [13C,15N]Ube2g1Y102G/Y104G, CSPs were measured upon titration with unlabeled ubiquitin. Although the ubiquitin binding properties of the bottom and L-side regions of the mutant of Ube2g1 were unchanged, the CSPs from the loop region completely disappeared (Fig. 4 B). Thus, the two Tyr residues are critical for non-covalent ubiquitin binding of the acidic loop in Ube2g1.

Introduction of Two Tyr Residues into the Ube2r1C Acidic Loop Increased Both Ubiquitin Binding Affinity and Lys-48-ubiquitylation Activity—We changed Gln-105–Ser-106–Gly-107 in Ube2r1C to Tyr-105–Gly-106–Tyr-107 (Ube2r1CYGY) to make its acidic loop more similar to that of Ube2g1 (12). The acidic loop in Ube2r1C is similar to the previously characterized weak ubiquitin-binding site 1 (UBS1) in the C-terminal tail of Ube2r1 (DLFYDDYYED) (26), which also consists of hydrophobic and acidic residues (Fig. 1). The T1, T2, and 1H,15N heteronuclear NOE values measured for Ube2r1C showed that the acidic loop region is more flexible than the bulk of the structured E2 core (data not shown); this finding is consistent with recent NMR 15N relaxation data for Ube2g2 (27).
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It has been reported that the C-terminal extension of Ube2r1 includes two weak ubiquitin binding motifs (UBS1, residues 205–214, and UBS2, residues 216–225) and an additional possibility of a third, very weak ubiquitin-binding site (residues 187–196) (26). However, Ube2r1C alone did not detectably bind to [15N]UB (data not shown); the low background with Ube2r1C made identification of ubiquitin binding differences between Ube2r1C and Ube2r1CYGY straightforward. Comparison of the HSQC spectra between Ube2r1C and Ube2r1CYGY showed that the effect of Tyr-105–Gly-106–Tyr-107 substitu-

tion was limited to the backbone chemical shifts only of the acidic loop residues (Fig. 5A). The CSP data showed that the acidic loop of Ube2r1CYGY, but not Ube2r1C, had a non-covalent interaction with ubiquitin (Fig. 5). However, the CSP magnitudes of the [15N]Ube2r1CYGY acidic loop were very small even in the presence of 4.5 mM ubiquitin, and thus the exact affinity for ubiquitin could not be determined. This low ubiquitin binding affinity together with the high Lys-48-ubiquitation activity of Ube2r1CYGY compared with Ube2r1C suggested a possible role for the acidic loop in an intramolecular interaction with E2 thioester-linked donor ubiquitin.

Synthesis of E2-UB Oxyester for NMR Experiments—To characterize the intramolecular interaction between the acidic loop and the E2-attached donor ubiquitin used for Lys-48-ubiquitylation, we tried to obtain more stable E2-UB complexes as E2-UB oxyester adducts. Crystal structures of the Ube2g1-UB oxyester formed by preparation of the Ube2r1CC93S-UBK48R and Ube2r1CC93S/YGY-UBK48R oxyesters. We also prepared chemically stable Ube2r1CY-[15N]UBK48R/G76C and Ube2r1CYGY-[15N]UBK48R/G76C disulfide adducts, as Ube2r1C contains only one Cys residue (i.e. Cys-93 at the active site). Interestingly, the HSQC spectra of disulfide-linked Ube2r1CYGY-[15N]UBK48R/G76C oxyster were greatly different from those of the Ube2r1CC93S- [15N]UBK48R oxyster (Fig. 7). The HSQC peaks from [15N]UBK48R in the oxysters were much less intense than those from the disulfide-linked [15N]UBK48R/G76C due to motion (i.e. conformational exchange) in an intermediate-to-slow NMR
barely identified for the Ube2r1C-[15N]UBK48R/G76C disulfide adducts where possible splittings in only a few peaks were This effect was much smaller or absent with the disulfide bic surface patch that includes residue Ile-44 of ubiquitin. How-

TABLE 1

| E2-UB oxyester | Half-life |
|-----------------|----------|
| Ube2g1C90S-[15N]UBK48R | 20.0 h |
| Ube2r1C93S/YGY-UBK48R | 20.0 h |
| Ube2g1C90S/Y102G/Y104G-UBK48R | 2.8 h |
| Ube2g1C90S-[15N]UBK48R | 8.8 ± 1.1 a |
| Ube2r1C93S/[15N]UBK48R | 5.8 ± 0.2 a |
| Ube2g1C90S/[15N]UBK48R | 6.4 ± 1.6 a |
| Ube2r1C93S/[15N]UBK48R | 2.8 ± 0.4 a |
| Ube2g1C90S/[15N]UBK48R | 24.3 ± 5.9 |
| Ube2r1C93S/[15N]UBK48R | 20.0 ± 6.5 |

a The donor ubiquitin was 13C/2H/15N-labeled; the hydrolysis rates (shown here as $t_\text{1/2}$) in buffer (pH 7.0-7.5, 50 mM HEPES and 100 mM NaCl) were determined from the several well separated HSQC peaks.

b The hydrolysis rates (i.e., $t_\text{1/2}$ values) at pH 8.0 were determined from two or more separate measurements using non-reducing SDS-PAGE analysis.

time scale that caused severe broadening and disappearance of the peaks. The ubiquitin CSPs of the oxyester relative to the chemical shifts of free [15N]UBK48R and also the CSPs of the disulfide adducts relative to free [15N]UBK48R/G76C showed that the [15N]UBK48R moiety of the oxyester had much larger CSPs than with the disulfide-linked [15N]UBK48R/G76C (Fig. 8, A and B). Many peaks of the Ube2r1C-[15N]UBK48R oxyester were split into two sets; these corresponded to the major hydrophobic surface patch that includes residue Ile-44 of ubiquitin. However, with the Ube2r1C/YGY-[15N]UBK48R oxyester, these resonances were not split and remained as one set (Fig. 7A). This effect was much smaller or absent with the disulfide adducts where possible splittings in only a few peaks were barely identified for the Ube2r1C-[15N]UBK48R/G76C disulfide but not for the Ube2r1C/YGY-[15N]UBK48R/G76C disulfide (Fig. 7B). Overall, evidence of intramolecular interaction between the donor ubiquitin and Ube2r1C was greatly reduced with the disulfide adduct relative to the oxyester adduct. A possible explanation is that, compared with the E2-UB oxyester linkage, the E2-UB disulfide is a poorer mimic of the physiological thioester (e.g., it is longer by approximately one S atom) and may interfere with the normal intramolecular interaction between the acidic loop and the donor ubiquitin.

The HSQC spectra indicated that the ubiquitin in the Ube2r1C/YGY-[15N]UBK48R oxyester has two different confor-

mations but that one conformation is preferred in the YGY-mutant complex (i.e., the Ube2r1C/YGY-[15N]UBK48R oxyester). The major set of peaks in the HSQC spectrum of the Ube2r1C/YGY-[15N]UBK48R oxyester displayed much larger CSPs than did the minor set, whereas the Ube2r1C/YGY-[15N]UBK48R oxyester displayed one set of peaks whose CSP magnitudes were similar to those of the minor set in the HSQC spectrum of the Ube2r1C/YGY-[15N]UBK48R oxyester (Fig. 8A). The absence of apparent non-covalent UB binding activity in Ube2r1C can explain why the donor ubiquitin exhibits (at least) two different conformations in its Ube2r1C/YGY-[15N]UBK48R oxyester form. Both weak intermolecular interactions can be entropy-stabilized in an intramolecular association. The intensified interactions of the donor ubiquitin with both the L-side and the acidic loop of Ube2r1C, by competing with each other, can produce two sets of HSQC peaks. The introduction of the two Tyr residues into the acidic loop of Ube2r1C likely shifts the equilibrium by stabilizing the interaction between the donor UB and the acidic loop. However, we cannot exclude a possibility that the preferred conformation is caused by other mechanisms, such as a conformational change accompanying binding. The chemical shift differences between Ube2r1C/YGY-[15N]UBK48R and Ube2r1C/YGY-[15N]UBK48R oxyesters showed that the interaction between the E2 and the hydrophobic patch of the donor ubiquitin was reduced more for Ube2r1C/YGY/[15N]UBK48R than for Ube2r1C/YGY-[15N]UBK48R oxyester (Fig. 8A).

Proposed Role of the Ube2g1 Acidic Loop for the Efficient Lys-48-ubiquitylation in the Absence of E3—We prepared Ube2g1-UB oxyesters to obtain more detailed information about the intramolecular interaction between Ube2g1 and the donor ubiquitin. Interestingly, the HSQC spectrum of the Ube2g1-[15N]UBK48R oxyester was well dispersed (Fig. 8A). Previous reports have shown that the [15N]Ube2d3~UB thioester mostly lost discernible HSQC peaks due to intermolecular interaction mediated by the attached donor ubiquitin and another [15N]Ube2d3~UB (31). The donor ubiquitin of the Ube2d3C85S/S22R-[15N]UBK48R oxyester has been found in an
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extended and dynamic state, referred to as an “open conformation,” that is not favorable for its ubiquitylation activity (30). Ube2g1 has UB binding surfaces similar to Ube2d3; in Ube2d3–UB, these promote the self-association of Ube2d3–UB. However, because there was no disappearance or significant decrease of HSQC peaks of the UB-oxyester when compared with [15N]Ube2g1, the [15N]Ube2g1 C90S–UB oxyester apparently lacks the intermolecular interaction.

Although we could not fully assign the HSQC peaks of the [15N]Ube2g1 C90S–UBK48R oxyester, comparisons of the HSQC and three-dimensional-HNCO spectra between [15N]Ube2g1 C90S–UBK48R oxyester and free [15N]Ube2g1 C90S made it possible to determine the interaction surface on Ube2g1 for the attached donor ubiquitin (Fig. 9). The HSQC peaks of the acidic loop and many other residues, especially those on the L-side, were changed too much to be traced reli-

FIGURE 7. HSQC spectra of Ube2r1CC93S- and Ube2r1CC93S/YGY-[15N]UBK48R oxyesters (A) and Ube2r1C- and Ube2r1C′-[15N]UBG76C disulfides (B). A, selected regions of the HSQC spectra show that the attached donor ubiquitin of the Ube2r1CC93S-[15N]UBK48R oxyester has two different conformations (top panel) but that of the Ube2r1CC93S/YGY-[15N]UBK48R oxyester has one predominant conformation (bottom panel). Residues labeled with an f are residues from ubiquitin that had dissociated from the E2. B, the same regions of the HSQC spectra of disulfide-linked Ube2r1C′-[15N]UBG76C and Ube2r1C′-[15N]UBK48R/G76C show that in both complexes the attached donor ubiquitin mostly has a single conformation and that the chemical shift differences from free ubiquitin are much smaller than with the oxyester complexes.

FIGURE 8. Chemical shift perturbations relative to free [15N]UB of [15N]UB resonances in the Ube2r1CC93S/YGY-[15N]UBK48R and Ube2r1C′-[15N]UBK48R oxyesters (A), Ube2r1C′-[15N]UBK48R/G76C disulfides (B), and Ube2g1 C90S–[15N]UBK48R and Ube2g1 C90S/Y102G/Y104G–[15N]UBK48R oxyesters (C). The chemical shifts of free [15N]UBK48R and [15N]UBK48R/G76C were used to determine the CSPs of E2–[15N]UBK48R oxyesters and disulfides, respectively. The chemical shift differences between the attached [15N]UB molecules of the wild-type and the acidic-loop mutant E2 are shown in the right panels. The negative bars represent the residues including prolines (Pro-19, Pro-37, and Pro-38) for which peaks were not shown in the HSQC spectra. The dotted bars in C (right panel) represent the residues apparent in the HSQC spectra of the Ube2g1 C90S–[15N]UBK48R oxyester but not the Ube2g1 C90S/Y102G/Y104G–[15N]UBK48R oxyester.
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ably from the spectrum of free [15N]Ube2g1C90S to the [15N]Ube2g1C90S-UBK48R oxyester (Figs. 9C and 10A). This result contrasts with Ube2d3C85S/S22R-[15N]UB oxyester (30) in which the chemical shift differences between the oxyester-bound and free [15N]UB were relatively small. To determine in more detail the binding mode of the donor ubiquitin in the Ube2g1-UB oxyester, we also prepared oxyester complexes of Ube2g1C90S and Ube2g1C90S/Y102G/Y104G using [13C,2H,15N]UBK48R. Due to their low stability in solution and larger chemical shift changes compared with TROSY peaks of free [13C,2H,15N]UBK48R, the 1H,15N TROSY peaks of Ube2g1C90S-UBK48R were assigned using additional TROSY-based three-dimensional-HNCO spectra. From time-dependent changes in the TROSY spectra, we also determined the half-lives of [15N]Ube2g1C90S- and [15N]Ube2g1C90S/Y102G/Y104G-[13C,2H,15N]UBK48R oxyesters to be 8.8 ± 1.1 and 5.8 ± 0.2 h, respectively (Table 1). The comparison of TROSY peaks from free and attached [13C,2H,15N]UBK48R showed that the hydrophobic patch of the attached donor ubiquitin interacts with the L-side of Ube2g1 (Fig. 8C).

The CSP-based Rosetta docking model of the acidic loop-deleted Ube2g1C90S-UBK48R oxyester together with additional fitting of the loop based on CSPs provided structural insight about the intramolecular interaction between the E2 acidic loop and the attached donor ubiquitin (Fig. 10). Although the exact modeling of the acidic loop structure in the Ube2g1C90S-UBK48R oxyester was difficult, the geometric constraint of the oxyester bond shows that the Gly-103 residues of the [15N]Ube2g1-UBK48R oxyester are likely to be located in the vicinity of Arg-74 of the donor ubiquitin. The active site of the Ube2g1C90S-UBK48R oxyester appears to be accessible only through the R-side of Ube2g1 because the L-side was completely occupied by the attached donor ubiquitin (Fig. 10E). Our data clearly supported the idea that an acceptor ubiquitin approaches the R-side of Ube2g1; this result agrees well with the recently reported docking model of Ube2S~UB thioester in which L-side positioning of the donor ubiquitin is important for the activity of Lys-11 ubiquitin-linkage formation (7). The chemical shift differences of [15N]UBK48R molecules between Ube2g1C90S-[15N]UBK48R and Ube2g1C90S/Y102G/Y104G-[15N]UBK48R oxyesters were much smaller than those between the free and attached [15N]UBK48R.

To estimate the $K_m$ values for ubiquitin in E3-independent ubiquitylation reactions by Ube2g1, Ube2g1Y104G, and Ube2r1CGYY, we measured the steady-state rates of diubiquitin formation as a function of ubiquitin concentration (Fig. 11).
The Km and kcat values for E3-independent ubiquitylation by Ube2k were reported previously to be 0.58 mM and 0.56 min−1, respectively (11); thus, ubiquitylation by Ube2k was used as a reference to validate our steady-state kinetic analysis. The Km value (0.5 mM) we determined for Ube2k agreed well with the reported value. Although we could not determine exact Km values due to a practical upper limit of several millimolar for the ubiquitin substrate in our assays, it was apparent that the Km for Ube2g1Y104G is at least severalfold greater than that of the wild-type Ube2g1 (Fig. 11). The Km data suggest that Tyr-104 in the acidic loop is important for the interaction of Ube2g1−UB thioester with an acceptor ubiquitin. Importantly, the HSQC peak of Gly-103 in the [15N]Ube2g1C90S, UBK48R oxyester form was shifted much less by the addition of free ubiquitin than that in [15N]Ube2g1 alone (Fig. 9A, inset), which suggests that the intermolecular interaction between the acidic loop and an acceptor ubiquitin in the context of the Ube2g1-UB oxyester is different from that with free Ube2g1.

Mutation in the Acidic Loop Region Changed the in Vitro Ubiquitylation Activity of Ube2g1—We introduced various point mutations into each ubiquitin-interaction site (bottom, S26R and L41A; L-side, E118R, I122A, and S126R; loop, Y104G, and L104G). The interaction surfaces between UBK48R and Ube2g1C90S mapped on a model of the oxyester complex. A, intramolecular UBK48R-interaction surfaces on Ube2g1C90S are highlighted on the structure of free Ube2g1. The red and yellow colors, respectively, indicate high and low magnitude CSPs. The residues in which the HSQC peaks shifted too much to be traced are shown in green (see Fig. 9C). B, Ube2g1C90S-interacting surface of UBK48R is highlighted using magenta, red, orange, and yellow colors to indicate high to low magnitude of CSPs, respectively. The residues colored black represent prolines and other residues that were invisible in the HSQC spectrum, and the green-colored residues indicate where resonances disappeared in the oxyester complex. C, the residues of Ube2g1C90S showing different chemical shifts between the oxyesters with Ube2g1C90S and Ube2g1C90S/Y102G/Y104G are highlighted as in panel B. D, the residues of Ube2g1 mutated for the ubiquitylation assays (Fig. 12) are shown superimposed onto a ribbon representation of the structure. E, surface presentation shows the donor UBK48R-occupied surface of the E2.

FIGURE 11. In vitro ubiquitylation by Ube2k, Ube2g1, Ube2g1Y104G, or Ube2r1CYGY in the presence of various concentrations of ubiquitin. A, the reaction of di-UB synthesis can be divided into two steps (I and Step-2). If the step-1 is faster than the step-2, the concentration of E2−UB is essentially constant (d[E2−UB]/dt = 0). The ubiquitylation activity of Ube2k and Ube2g1 was assessed at pH 7.5 in the presence of increasing concentrations of E1 and with 2.4 mM ubiquitin. The E1 concentration used was ~1.5 μM for further steady-state kinetic analysis of E3-independent ubiquitylation. AU, absorbance units. B, the in vitro ubiquitylation reactions were performed in the presence of increasing concentration of the substrate ubiquitin with fixed concentrations of E1 and E2. The amount of the diubiquitin product bands increased as the concentration of the substrate ubiquitin increased. C, the Km values of E2-mediated ubiquitylation were estimated for Ube2k, Ube2g1, Ube2g1Y104G, and Ube2r1CYGY from the modified Michaelis-Menten equation (see “Experimental Procedures”). The Km value of Ube2K, Ube2g1, or Ube2r1YGY was 0.5 ± 0.06, 2.5 ± 0.7, or 2.9 ± 0.7 mM, respectively. The Km value of Ube2g1Y104G was too high to be determined by this experiment.

The Km and kcat values for E3-independent ubiquitylation by Ube2k were reported previously to be 0.58 mM and 0.56 min−1, respectively (11); thus, ubiquitylation by Ube2k was used as a reference to validate our steady-state kinetic analysis. The Km value (~0.5 mM) we determined for Ube2k agreed well with the reported value. Although we could not determine exact Km values due to a practical upper limit of several millimolar for the ubiquitin substrate in our assays, it was apparent that the Km for Ube2g1Y104G is at least severalfold greater than that of the wild-type Ube2g1 (Fig. 11). The Km data suggest that Tyr-104 in the acidic loop is important for the interaction of Ube2g1−UB thioester with an acceptor ubiquitin. Importantly, the HSQC peak of Gly-103 in the [15N]Ube2g1C90S, UBK48R oxyester form was shifted much less by the addition of free ubiquitin than that in [15N]Ube2g1 alone (Fig. 9A, inset), which suggests that the intermolecular interaction between the acidic loop and an acceptor ubiquitin in the context of the Ube2g1-UB oxyester is different from that with free Ube2g1.

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Y102G/Y104G, and E99G/D100G) to explore their effects on the ubiquitylation activity. Our rationale for choosing these mutations is as follows. Mutations in specific Ube2g1 residues (S26R and L41A in the bottom; E118R in the L-side) were determined from the sequence alignment with Ube2r1 (Fig. 1), because Ube2r1C did not display any detectable non-covalent ubiquitin binding. Ser-26 corresponds to Ser-22 of Ube2d3 (UbcH5c), and Ube2d3S22R greatly decreases the ubiquitin binding activity (31). The I122A mutation corresponds to the L125A mutation of Ube2S; it was reported that Leu-125 is important for Lys-11-ubiquitylation activity by positioning the donor ubiquitin into the L-side of Ube2S (7). The results in Fig. 12 show that only the mutations in the acidic loop resulted in severe loss of Lys-48-ubiquitylation activity; none of the mutations in the bottom or L-side of Ube2g1 had significant defects in the ubiquitylation activity. The lower ubiquitylation activity did not result from an impaired formation of E2–UB thioester by E1 enzyme (Fig. 12, bottom panel). We additionally confirmed that the time-course activations of Ube2g1, Ube2g1S126R, and Ube2g1Y104G by E1 enzyme were almost identical, and their productions of diubiquitin species were gradually increased as increasing the incubation time from 0 to 2 h (data not shown).

The activity of the Y104G mutant was lower than that of the wild type, and the Y102G/Y104G mutant was nearly inactive. Other acidic loop mutations in Ube2g1 (i.e. E99G/D100G) also abolished ubiquitylation activity, which is in agreement with a previous report that showed decreased activity in the acidic loop mutants of yeast Cdc34 in the presence of Skip/Cullin/F-box-E3 (10). Residues Glu-99 and Asp-100 are located in the R-side of Ube2g1 (Fig. 10D). Interestingly, the ubiquitylation reaction catalyzed by Ube2g1E99G/D100G produced additional unidentified protein bands (shown by an asterisk in Fig. 12); these most likely reflect autoubiquitylation of the E2 itself.

Recent NMR studies of Ube2g2 proposed that His-94, which is positioned close to Cys-89 in the active site, is likely to have a catalytic role (27). His-94 corresponds to His-95 of Ube2g1 and is also conserved in Ube2r1 (Fig. 1). We confirmed that this His residue, like the Asn-82 residue that is evolutionarily conserved among E2 proteins and is important for the formation of a ubiquitin-protein linkage (32), is important for Lys-48-ubiquitylation activity. Either H95A or N82A mutants of Ube2g1 completely eliminated the ubiquitylation activity (Fig. 12). Other Ube2g1 His-95 mutants (H95R and H95E) similarly failed to show detectable activity (data not shown).

DISCUSSION

We have shown that the intramolecular interaction between the acidic loop of Ube2g1 and the donor ubiquitin plays an important role in the Lys-48-ubiquitylation activity of E2 enzymes. Ubiquitin binding properties of the acidic loop were investigated with two E2–UB thioester mimics, E2-UB oxyester, E2-UB oxyster, and disulfides, and then the inferences regarding E2 Lys-48-ubiquitylation activity were confirmed by enzymatic assays using various point mutants. The Ube2g1-UB oxyster can serve as a functional analog of the labile E2–UB thioester intermediate. Ube2g1C90S and Ube2r1C90S/Y95S/AC28 were able to produce Lys-48 diubiquitin.

The presence of the cognate RING-E3 ligase restricts the dynamics of the donor ubiquitin in the Ube2d3-UB oxyster to a position (closed conformation) that is more favorable for the ubiquitylation reaction (33). Moreover, donor ubiquitins on Ube2S (7), Ubc1 (the yeast homolog of Ube2k) (22), and Ube2r1
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(23) have been proposed to adopt closed conformations predicted to facilitate ubiquitylation. We have shown here that the donor ubiquitin in the Ube2g1-UB oxyester interacts with the L-side of Ube2g1 and thus adopts a closed conformation that is similar to the models suggested for Ube2S, Ubc1, and Ube2r1. However, adoption of the closed conformation by the donor ubiquitin through its interaction with the L-side of E2 does not seem to be critical for Lys-48-ubiquitylation by Ube2g1. The donor ubiquitin in Ube2g1C90S/Y102G/Y104G-UBK48R oxyester also can adopt the closed conformation, although no ubiquitylation activity was observed for the Ube2g1C90S/Y102G/Y104G mutant. Moreover, the introduction of two Tyr residues into the acidic loop of Ube2r1C stabilized a conformation of the donor ubiquitin that, according to NMR CSP data, reflected a decreased interaction between the hydrophobic patch of the donor ubiquitin and the L-side of Ube2r1C<sub>GYG</sub> (Fig. 8A). Although both Ube2g1C90S and Ube2r1C<sub>GYG</sub> displayed appreciable Lys-48-ubiquitylation activity, the configurations of their donor ubiquitin in the oxyester forms appeared to differ. Smaller CSPs (relative to free [15N]UBK48R) with the Ube2r1C<sub>GYG</sub>-[15N]UBK48R oxyester than with the Ube2g1C90S-[15N]UBK48R oxyester indicate reduced intramolecular interaction between the L-side of Ube2r1C<sub>GYG</sub> and the hydrophobic patch of the donor ubiquitin. For Ube2g1 or Ube2r1C<sub>GYG</sub> Lys-48-ubiquitylation activity, it is likely that the local structure formed by the acidic loop and the C-terminal region of the attached donor ubiquitin is more important than the simple positioning of the donor ubiquitin onto the L-side of the E2. These results suggest that Ube2g1, Ube2r1, and Ube2k, which are known to generate Lys-48-linked polyubiquitin efficiently without E3, seem to use a different mechanism to conjugate acceptor and donor ubiquitin. Other evidence supporting this idea is that the UB<sub>N60A</sub> mutant displayed different Lys-48-ubiquitylation activities as an acceptor for Ube2k or Ube2g1, showing a severe defect with Ube2k, whereas ubiquitylation by Ube2g1 was relatively unaffected by the ubiquitin N60A mutation (12).

In the context of Ube2g1-UB oxyester, the acidic loop mutation (Y102G/Y104G) changes the chemical environment of the C-terminal region and the edge of the hydrophobic patch in the attached ubiquitin (Fig. 8C and Fig. 10C). The estimated K<sub>n</sub> values of Ube2g1 and Ube2g1<sub>Y102G</sub> showed that the acidic loop mutation also affected recruitment of the acceptor ubiquitin (Fig. 11). Moreover, production of a tiny amount of diubiquitin with linkage(s) other than to Lys-48 was identified with the E99G/D100G mutation (Fig. 12, see gel band indicated as D<sub>IP</sub>); this suggests that the acidic loop also may play an important role in proper alignment of an acceptor ubiquitin. The production of non-Lys-48-linked diubiquitin was reported for Ube2d2 and Ube2b in the absence of E3 (8).

The acceptor ubiquitin can approach the active Cys residue via the R-side of Ube2g1-UB thioester. Recently Ube2g2, a homolog of Ube2g1, was shown to form a functional dimer, and that residue Cys-48 residue is important for the dimerization (34). Because the Cys-48 residue is not conserved in Ube2g1 (Fig. 1) and Ube2g1 ubiquitylation activity is independent of the E2 concentration (Fig. 3), the mechanisms of ubiquitylation between Ube2g1 and Ube2g2 would be different from each other. The cognate E3 ligase(s) of Ube2g1 has not yet been identified; therefore, we have fit our model structure of the Ube2g1-UB oxyester (Fig. 10) to the Ube2g2-UB<sub>78-E3</sub> complex structure reported by Das et al. (35). A fit was obtained without any steric clash and in which the R-side of Ube2g1, the candidate surface for an acceptor ubiquitin, is well exposed to solvent (not shown).

Interestingly, the recent docking model of Ube2r1~UB thioester that was based on the Rosetta force field (and does not contain the acidic loop or the C-terminal extension) shows that the donor ubiquitin is located at the L-side region of Ube2r1, which is similar to our model for the Ube2g1C90S/Y102G/Y104G oxyester, where Ile-44 of the donor ubiquitin is close to Ser-129 of Ube2r1, and thus the I44A mutation of the donor ubiquitin can be suppressed by the S129L mutation of Ube2r1 (23). The introduction of two Tyr residues in the acidic loop of Ube2r1C stabilized the interaction between the donor ubiquitin and the L-side of Ube2r1C<sub>GYG</sub> but increased the Lys-48-ubiquitylation activity. The acidic loop of Ube2r1 displayed a lower binding affinity to the donor ubiquitin than did that of Ube2g1, and thus the acidic loop and the C-terminal tail of Ube2r1 might work together during the catalysis. It might be possible that the residues 184–196 of Ube2r1 stabilize the conformation of the donor ubiquitin in a manner similar to this Ube2r1~UB thioester docking model (23) and thus facilitate the recruitment of an acceptor ubiquitin. Ube2r1 could adapt to its specific function in the presence of SCE-E3 by losing the ubiquitin binding property of its acidic loop.

Acidic loop or tail motifs are known to be important for the ubiquitylation activities of E2 or E3 enzymes; examples are the acidic loops in Ube2g1 and Ube2g2 (36), the acidic tail (residues 888–900) in human Nedd4 (HECT-E3) (37), and both acidic loop and acidic tail in Ube2r1 (10, 26, 38). These motifs possess both acidic and bulky hydrophobic residues and usually are near the active-site Cys residue. Although the acidic tail of Ube2r1 is far from the active site, it is known to interact with a donor ubiquitin of Ube2r1 (26, 39). Although the ubiquitin binding of the acidic tail of Nedd4 has not yet been identified, the others in Ube2g1 and Ube2r1 display a weak ubiquitin binding activity. HECT-E3 ligase could be similar with E2 enzyme, because the active Cys residue forms a labile thioester bond with a donor ubiquitin. We speculate that, as with the acidic loop of Ube2g1, the acidic tail of Nedd4 may interact with donor ubiquitin to enhance its ubiquitylation activity.

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