Certain Pairs of Ubiquitin-conjugating Enzymes (E2s) and Ubiquitin-Protein Ligases (E3s) Synthesize Nondegradable Forked Ubiquitin Chains Containing All Possible Isopeptide Linkages*

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It is generally assumed that a specific ubiquitin ligase (E3) links protein substrates to polyubiquitin chains containing a single type of isopeptide linkage, and that chains composed of linkages through Lys48, but not through Lys63, target proteins for proteasomal degradation. However, when we carried out a systematic analysis of the types of ubiquitin (Ub) chains formed by different purified E3s and Ub-conjugating enzymes (E2s), we found, using Ub mutants and mass spectrometry, that the U-box E3, CHIP, and Ring finger E3s, MuRF1 and Mdm2, with the E2, UbcH5, form a novel type of Ub chain that contains all seven possible linkages, but predominantly Lys48, Lys63, and Lys11 linkages. Also, these heterogeneous chains contain forks (bifurcations), where two Ub molecules are linked to the adjacent lysines at Lys6 or Lys11, Lys27 + Lys29, or Lys29 + Lys31 on the preceding Ub molecule. However, the HECT domain E3s, E6AP and Nedd4, with the same E2, UbcH5, form homogeneous chains exclusively, either Lys48 chains (E6AP) or Lys63 chains (Nedd4). Furthermore, with other families of E2s, CHIP and MuRF1 synthesize homogeneous Ub chains on the substrates. Using the dimeric E2, UbcH13/Uev1a, they attach Lys63 chains, but with UbcH1 (E2–25K), MuRF1 synthesizes Lys48 chains on the substrate.

We then compared the capacity of the forked heterogeneous chains and homogeneous chains to support proteasomal degradation. When troponin I was linked by MuRF1 to a Lys48-Ub chain or, surprisingly, to a Lys63-Ub chain, troponin I was degraded rapidly by pure 26S proteasomes. However, when linked to the mixed forked chains, troponin I was degraded quite poorly, and its polyUb chain, especially the forked linkages, was disassembled slowly by proteasome-associated isopeptidases. Because these Ring finger and U-box E3s with UbcH5 target proteins for degradation in vivo, but Lys63 chains do not, cells probably contain additional factors that prevent formation of such nondegradable Ub-conjugates and that protect proteins linked to Lys63-Ub chains from proteasomal degradation.

In eukaryotic cells, ubiquitination serves to target regulatory and misfolded proteins for rapid degradation by proteasomes (1–3), to trigger endocytosis of membrane proteins (4), and also to allow specific protein–protein associations important in signal transduction, DNA repair, and gene transcription (5–8). Protein ubiquitination involves formation of isopeptide linkages between the C-terminal carboxyl group of a ubiquitin (Ub) and an e-amino group on a lysine on the protein substrate or a preceding Ub to form a polyUb chain. To synthesize such linkages, the C-terminal carboxyl group of a Ub is first activated by formation of a thioester bond with a cysteine on the Ub-activating enzyme (E1), and the activated Ub is then transferred as a thioester to one of the 20–40 Ub-conjugating enzymes (E2) of the cell. The formation of a Ub chain on the substrate is then catalyzed by a Ub ligase (E3), which binds the substrate and an E2. Several families of E3s exist that differ in structure and mechanism. If ubiquitination is catalyzed by a member of the Ring finger or the U-box family, the activated Ub is transferred from the E2 directly to a lysine on the protein substrate or to a preceding Ub. The abundant Ring finger and the related U-box families are small monomeric proteins that bind the substrate at one end and then in that vicinity release the reactive Ub from the E2-Ub thioester (9, 10). If ubiquitination is catalyzed by an E3 of the HECT domain family, the activated Ub is transferred from the E2 first to a cysteine on the E3 to form another thioester bond and then to the substrate or to a preceding Ub.

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4 The abbreviations used are: Ub, ubiquitin; UPK, ubiquitin peptide modified at lysine n by ubiquitination; Forked chain, Ub chain in which two Ub chains are linked to the adjacent lysines on the preceding Ub; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; LC-MSMS liquid chromatography-tandem mass spectrometry; SIM, selective ion monitoring.
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(11). The much larger multicomponent SCF complexes, SOCS E3s and APC/C, contain a substrate-binding subunit and a distinct Ring finger subunit, which catalyzes direct addition of a Ub from the E2 to the substrate.

Because a Ub molecule contains seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63), there are seven different possible types of isopeptide linkage. It is generally assumed that Ub chains contain only one type of isopeptide linkage (12–14), and that the nature of the Ub linkage determines the specific fate of the protein. Most proteins degraded by 26S proteasomes are believed to be linked to a homogeneous polyUb chain, in contrast to the fate of the protein. Most proteins degraded by 26S proteasomes and that the nature of the Ub linkage determines the specific possible types of isopeptide linkage. It is generally assumed that Lys63 which the Ubs are linked through other lysines are believed to serve roles unrelated to proteolysis (3–5, 7). For example, Lys63 chains have been shown to function in DNA repair (7) and signal transduction (5). However, the isopeptide linkages in polyUb chains formed by different types of purified ubiquitinat- ing enzymes have not been studied systematically.

Most conclusions about the nature of the Ub chain have been based on qualitative studies using various Ub mutants in vivo or in vitro, and in only a few cases has the validity of these conclusions been confirmed by mass spectrometry (16, 17). In fact, it has not yet been established for most E3s whether they synthe- size Ub chains composed of single or multiple types of linkages or whether the nature of the Ub linkage is determined by the E2 or the E3. Such information is essential to understand the func- tions of the many different types of Ub ligases of the cells and the consequences of protein ubiquitination.

In this study, we examined systematically the nature of the isopeptide linkages in the polyUb chains formed by different types of E2s and E3s that are known to target proteins for degrad- ation. These initial studies uncovered several unexpected features of the ubiquitination process as follows: 1) the type of isopeptide linkages formed by U-box or Ring finger E3s is deter- mined by the E2, and a single E3 can form different types of chains depending on the E2; 2) the small Ring finger and U-box E3s, using UbcH5 as the E2, form novel types of Ub chains that contain all seven type of isopeptide linkages and that are also forked (i.e. they contain two UbS linked to a preceding Ub); 3) by contrast, different HECT domain E3s using this same E2, UbcH5, can form homogeneous chains composed of different linkages. These findings enabled us to investigate the capacity of these different types of conjugates to be degraded by pure 26S proteasomes. We found that purified 26S proteasomes degrade very poorly the proteins linked to these novel, forked Ub chains composed of mixed linkages, although they efficiently degrade proteins linked to Lys48 chains and also surprisingly to Lys63 chains. These observations demonstrate important differences between the functioning of different E2-E3 pairs, and they clearly do not support certain widely accepted conclusions about the nature of Ub chains capable of supporting protein degradation by the Ub-proteasome pathway.

EXPERIMENTAL PROCEDURES

Ubiquitination and Proteasomal Degradation—Ubiquitina- tion of luciferase was assayed by a modification of the method of Murata et al. (18). Luciferase (75 nM) was heat-treated to 43 °C for 10 min in the presence of Hsp70 (150 nM) and then cooled on ice for 5 min. The Hsp70-luciferase complex was added to the ubiquitination mixture containing His6-E1 (5 nM), E2 (750 nM), His6-ChIP (300 nM), bovine ubiquitin (58 μM) in the buffer containing 20 mM Tris-Cl (pH 7.6), 20 mM KCl, 5 mM MgCl2, 2 mM ATP, and 1 mM dithiothreitol. The reactions were run for 60 min at 37 °C. Ubiquitination of troponin I by MuRF1, HHR23A by E6AP, and autoubiquitination of GST-MuRF1, Mdm2, GST-E6AP, or GST-Nedd4 were carried out under these same conditions but without the heat treatment or Hsp70. The autoubiquitination of Cdc34 was carried out with yeast E1 and Ub in the same buffer at 30 °C. For the ubiquitina- tion of Sic1, 0.5 pmol of SIC, Cdc34, and 70 pmol of Cdc34A were incubated with 5 pmol of Sic1 at 37 °C for 1 h. Ub-luciferase conjugates were resolved on 4–12% gradient SDS-PAGE and detected by PhosphorImager. Ubiquitinated MuRF1, Mdm2, E6AP, Nedd4, and Cdc34 were detected by Coomassie Blue staining. Also ubiquitinated Sic1 was detected by silver staining.

Proteasomal degradation of ubiquitinated troponin I was assayed by adding purified rabbit muscle 26S proteasomes (4 nM) to the reaction mixture at the onset of the ubiquitination. Data shown are the average of three separate experiments.

Mass Spectrometry—Ubiquitinated luciferase or HHR23A were immunoprecipitated with antibody, resolved on 4–12% SDS-PAGE, and stained with Coomassie Blue. Ubiquitinated MuRF1, Mdm2, E6AP, HHR23A, Nedd4, Cdc34, and Sic1 were resolved on SDS-PAGE without immunoprecipitation. Ub-conjugate bands larger than 188 kDa were excised from the SDS-polyacrylamide gels and digested with sequencing grade trypsin (Promega). When required, sequencing grade Arg-C, Glu-C, and Asp-N were used instead of trypsin. Digested samples were loaded onto a fused silica microcapillary C18 column (Magic: Michrom Bioresources, Auburn, CA) prepared in-house (75 μm inner diameter, 10 cm long). An Agilent 1100 high pressure liquid chromatography system was used to deliver a gradient across a flow splitter to the column over 40 min. The eluent was directed into an LCQ-Deca electrospray ion-trap mass spectrometer (ThermoFinnigan), and the eluent peptides were dynamically selected for fragmentation by the operating software. The MS-MS data were analyzed using SEQUEST data base search tool. All matched peptides were confirmed by visual examination of the spectra, and all spectra were searched against the Ub peptide data base. Modifi- cation was permitted to allow for the detection of the follow- ing (mass shift shown in daltons): oxidized methionine (+16) and ubiquitinated lysine (+114). All data shown were obtained in at least two separate experiments. We defined specific UPKs as highly abundant when the frequency of identification of the UPK was higher than 10% of the total identified UPKs, and if their abundance was less than 10%, they were defined as of low abundance.

Ubiquitin Peptide Enrichment Analysis—Luciferase was ubiquitinated as described above and then purified using the antibody against luciferase. One-half was kept as a control, and the other half was incubated with 26S proteasomes. To analyze deubiquitination, the ubiquitinated luciferase attached to the bead was incubated with 0.45 μg of rabbit muscle 26S protea- somes (19) for 1 h at 37 °C. The quantity of each UPK was...
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CHIP and Monomeric Ring Finger E3s Form Ub Chains Containing All Possible Isopeptide Linkages—We initially studied the nature of the isopeptide linkages formed by CHIP, a U-box E3, which functions with Hsp70 in the selective degradation of misfolded proteins (20), including several proteins important in human disease (e.g. cystic fibrosis transmembrane regulator (21) and phosphorylated Tau (22)). With E1, UbCH5 as the E2, Hsp70, and Ub, CHIP catalyzed ligation of multiple Ub molecules to the model substrate, heat-denatured luciferase (18) (Fig. 1A). To identify the isopeptide linkages in these chains, we used various single-lysine Ub mutants, each of which contained only one lysine residue with the six other lysines replaced by arginines (12). Surprisingly, all seven mutant UbS supported the formation of a polyUb chain, although with quite different efficiencies (Fig. 1A). Wild type Ub supported the synthesis of the longest polyUb chain, but long chains were also formed with Ub mutants containing a single-lysine at Lys57, Lys63, Lys48, or Lys63. Thus CHIP can form all seven possible isopeptide linkages (in sharp contrast to a prior report (23)).

Because luciferase has multiple lysine residues, a large shift in molecular weight upon Ub conjugation can occur through ligation of multiple Ub molecules to different sites on the substrate or by formation of a single long polyUb chain. However, CHIP linked to luciferase only with a single methylated Ub, which lacks free amino groups and cannot form a Ub chain (Fig. 1B). Therefore, CHIP can attach a Ub to only a single site on luciferase, and the large conjugates formed with wild type Ub are because of a single long polyUb chain on that site.

To test whether other monomeric E3s can also form all types of isopeptide linkages, we used single-lysine and K48R substitution Ub mutants and assayed chain formation during autoubiquitination by two medically important members of the large Ring finger family of E3s, MuRF1, the muscle-specific E3 critical in muscle atrophy (24), and Mdm2, the oncoprotein that catalyzes ubiquitination of the tumor suppressor p53 (25). Like CHIP, MuRF1 (Fig. 1C) and Mdm2 (data not shown) with UbCH5 as the E2 could synthesize polyUb chains using any of the single-lysine Ub mutants. Thus, they can use any lysine residue in chain formation. These Ring finger E3s also showed no requirement for the Lys48, which is widely believed to be essential for proteasomal degradation. In fact, with the K48R mutant, MuRF1 formed long polyUb chains (Fig. 1C, 8th lane), similar in length to those formed with wild type Ub (1st lane). To test whether MuRF1 and UbCH5 also ubiquitinated exogenous substrates, we assayed the ubiquitination of troponin I using all seven types of isopeptide linkages (26) (Fig. 1D). All seven single-substitution Ub mutants, each of which contained one substitution of lysine with arginine, like wild type Ub, supported the polyubiquitination of troponin I (as was also found with autoubiquitination of MuRF1). Thus, with UbCH5 two types of ubiquitinating enzymes can form polyUb chains containing all possible isopeptide linkages and do not show any preference for Lys48 linkages.

Mass Spectrometry Confirms Multiple Types of Isopeptide Linkages in These PolyUb Chains—To eliminate the possibility that these various Ub mutants alter the types of linkages formed, we investigated the types of isopeptide linkages formed with wild type Ub by nano-LC-MSMS (27). After ubiquitination of luciferase by CHIP with wild type Ub, the Ub-conjugates were purified with an anti-luciferase antibody and subjected to exhaustive tryptic digestion. Cleavage by trypsin released from the Ub chains a large variety of peptides that were identified by mass spectrometry. Each type of isopeptide linkage yields a distinct peptide (termed a UPK) composed of the C-terminal Gly of one Ub linked to the ε-amino group of a lysine plus the neighboring residues in the proximal Ub (Fig. 2A and supplemental Fig. S3). Mass spectrometry of these UPKs also demonstrated that CHIP formed polyUb chains containing all seven possible isopeptide linkages (Table 1), as suggested by the observations with Ub mutants (Fig. 1A).

The relative amounts of UPKs indicative of linkages to Lys57, Lys11, Lys48, or Lys63 were determined based on the number of spectra assigned to the corresponding UPKs (not on their peak intensities). This spectral sampling is indicative of the relative abundance of peptides (28). UPK48 and UPK63 were found analyzed by nano-SIM, and the data from three separate experiments were averaged.

RESULTS

CHIP and Monomeric Ring Finger E3s Form Ub Chains Containing All Possible Isopeptide Linkages—We initially studied the nature of the isopeptide linkages formed by CHIP, a U-box E3, which functions with Hsp70 in the selective degradation of misfolded proteins (20), including several proteins important in human disease (e.g. cystic fibrosis transmembrane regulator (21) and phosphorylated Tau (22)). With E1, UbCH5 as the E2, Hsp70, and Ub, CHIP catalyzed ligation of multiple Ub molecules to the model substrate, heat-denatured luciferase (18) (Fig. 1A). To identify the isopeptide linkages in these chains, we used various single-lysine Ub mutants, each of which contained only one lysine residue with the six other lysines replaced by arginines (12). Surprisingly, all seven mutant UbS supported the formation of a polyUb chain, although with quite different efficiencies (Fig. 1A). Wild type Ub supported the synthesis of the longest polyUb chain, but long chains were also formed with Ub mutants containing a single-lysine at Lys57, Lys63, Lys48, or Lys63. Thus CHIP can form all seven possible isopeptide linkages (in sharp contrast to a prior report (23)).

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FIGURE 1. The purified ubiquitin ligases, CHIP and MuRF1, with UbCH5 can form polyubiquitin chains containing all isopeptide linkages. Autoubiquitination or ubiquitination of substrate proteins were performed in the presence of recombinant E1, UbCH5, ATP, and wild type (WT) or mutant Ub indicated. Each of these gels were typical of results obtained in two or three independent experiments. A, CHIP can form a polyUb chain on luciferase with any single-lysine mutant of Ub (3rd to 9th lanes) as shown by Western blotting with an anti-luciferase antibody. Ubiquitination reactions were performed in the presence of recombinant human E1, UbCH5, Hsp70, ATP, and wild type Ub, methylated Ub, or different Ub mutants. No change in substrate migration was observed without Ub (1st lane) or without addition of the E3 (data not shown). B, although a polyUb chain was formed with wild type Ub (1st lane), only mono-ubiquitinated luciferase was formed with methylated Ub (MeUb, 2nd lane). CHIP can form a polyUb chain only at a single site on luciferase. Firefly luciferase was ubiquitinated by pure CHIP with UbCH5 as the E2. Ubiquitinated luciferases were detected by Western blotting with an anti-luciferase antibody. C, autoubiquitination of MuRF1 with wild type Ub (1st lane), single-lysine mutant (2nd to 7th lanes) of Ub, as well as K48R point mutant (8th lane). Ubiquitin conjugates were detected by Coomassie Blue staining. No conjugation was observed without Ub (9th lane). Further evidence for formation of mixed chains containing all linkages was obtained by mass spectrometry of conjugates formed with wild type Ub (Table 1). D, human cardiac troponin I was ubiquitinated by MuRF1 and UbCH5 using wild type Ub (2nd lane) and all single-substitution Ub mutants (4th to 10th lanes). Methylated Ub (3rd lane) supported only mono-ubiquitination of troponin I. Ubiquitination of troponin I was detected by Western blotting with an anti-troponin I antibody.

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formation with Lys48 isopeptide linkages (supplemental Fig. 3. Single letter symbols of amino acid residues identified by trypsin of a peptide indicating the whole length of UPK6/11 is presented in description in [49x317]luciferases formed by CHIP and UbcH5 were sequenced by tandem MS as described in [49x317]isopeptide linkages containing Lys48 linkages, even though UbcH5 is used as contrast, the HECT domain E3s form polyUb chains through homogeneous chains are linked to adjacent lysines on the proximal Ub molecules. By contrast, when the ubiquitination is mediated by U-box or Ring finger E3s and UbcH5, polyUb chains is formed through mixed type of isopeptide linkages and even contain forks where two Ub molecules were linked covalently to adjacent lysines on the proximal Ub molecule at Lys6 and Lys11 that indicated that two Ub molecules were linked covalently to adjacent lysines when those act with Cdc34 or UbcH13/Uev1a, respectively. E2. Also Ring finger E3 (SCF complex) and U-box E3 (CHIP) form homogeneous linkages when those act with Cdc34 or UbcH13/Uev1a, respectively.

most frequently, whereas UPK6 was rarely found. It is noteworthy that the most abundant linkages (UPK11, UPK48, and UPK63) were reported as the most abundant UPKs in analysis of the total Ub-conjugates in growing yeast (27). In our experiments, UPK27, UPK29, and UPK33 were also formed but could not be quantitated reliably by mass spectrometry, because the sequences from which they were derived were digested only partially, even after exhaustive trypsinization. To verify the findings obtained with trypsin, the ubiquitinated luciferase was incubated with a mixture of other endoproteases (Arg-C, Glu-C, and Asp-N), and the Ub peptides were identified by nano-LC-MSMS (Table 1). Again peptides corresponding to all seven possible isopeptide linkages were identified, but they were generated with lower efficiencies than with trypsin. However, with the multiple protease treatment, we were able to identify reliably UPK27, UPK29, and UPK33. Interestingly with both protease treatments, no peptide was found indicating N-terminal ubiquitination.

We then tested whether the Ring finger E3s with UbcH5 and wild type Ub formed Ub chains containing all possible isopeptide linkages. After autoubiquitination, the MuRF1 and Mdm2 conjugates were treated with trypsin, and the released peptides were analyzed by nano-LC-MSMS. Again, all seven possible UPKs were found, and their relative abundance appeared similar in the Mdm2 and MuRF1 conjugates and resembled that in luciferase ubiquitinated by CHIP. In Mdm2 during autoubiquitination (Table 1) also revealed the presence of UPK6/11 forked peptides, whose relative abundance resembled that in luciferase ubiquitinated by CHIP. In addition, when the conjugates produced by CHIP, MuRF1, and Mdm2 were digested by trypsin, these analyses demonstrated peptides indicative of additional types of ubiquitin conjugates by trypsin.
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**FIGURE 3.** CHIP or MuRF1 with UbcH13/Uev2a dimer as the E2 forms polyUb chains through Lys63 linkages. Autoubiquitination or ubiquitination of substrate proteins was performed in the presence of recombinant E1, UbcH13/Uev1a, ATP, and wild type (WT) or mutant Ub as indicated. Each of these gels was typical of results obtained in two or three independent experiments. A, CHIP, with UbcH13-Uev1a complex, can form Ub chains through Lys63 linkages. Wild type Ub (2nd lane) and all single-substitution Ub mutants, except K63R Ub (11th lane), supported the formation of polyUb chains. Methylated Ub (MeUb, 3rd lane) and no lysine Ub (K0, 4th lane) did not form any Ub-conjugates or anchorless Ub chains. Proteins were detected using SDS-PAGE and Coomassie Blue staining. B, CHIP and UbcH13/Uev1a can ubiquitinate denatured luciferase through only Lys63 linkages. The luciferases from reactions in A were analyzed by Western blotting (upper panel). Single-substitution Ub mutants, except K63R Ub, supported the ubiquitination of luciferase. K63R Ub, methylated Ub, and no lysine Ub (K0) failed to form a Ub chain on luciferase. Unlike luciferase, CHIP (middle panel) or Hsp70 (lower panel) was not ubiquitinated at all. All proteins were detected by Western blotting. C, MuRF1 with UbcH13/Uev1a can form Ub chains through Lys63 isopeptide linkages. The anchorless Ub chains and Ub-conjugates formed by MuRF1 and UbcH13/Uev1a were analyzed by SDS-PAGE and Coomassie Blue staining. Wild type Ub and single-substitution Ub mutants except K63R Ub (10th lane) supported the ubiquitination. D, K63R Ub mutant supports the ubiquitination of troponin I by MuRF1 and UbcH13/Uev1a with low processivity. Using reactions in C, ubiquitinated troponin I was detected by Western blotting. Wild type Ub (2nd lane) and K11R, K29R, K33R, and K48R Ub mutants supported the extensive ubiquitination of troponin I. Troponin I was mono-ubiquitinated with methylated Ub (3rd lane), and K6R and K27R supported limited ubiquitination. Ubiquitination with K63R generated large amount of short Ub-troponin I conjugates (10th lane), but the processivity is low compared with wild type or other mutants. E, troponin I is ubiquitinated by MuRF1 and UbcH13/Uev1a through Lys63 isopeptide linkages. Ubiquitination of troponin I by MuRF1 and UbcH13/Uev1a with wild type (2nd lane) or single-lysine Ub mutants (4th to 10th lanes) was analyzed by Western blotting. Only Lys63 single-lysine Ub mutant as well as wild type Ub supported the formation of large Ub-troponin I conjugates. Other single-lysine Ub mutants supported ubiquitination of troponin I with low processivity (4th to 9th lanes).

Fig. S3). The formation of UPK6/11, UPK27/29, and UPK29/33 was verified by mass spectrometry after the polyUb chains were digested by the mixture of proteases, Arg-C, Glu-C, and Asp-N (Table 1). However, quantitation of UPK27/29 and UPK29/33 was less reproducible than that of UPK6/11, because these forked peptides are not released quantitatively upon trypsin digestion.

It is noteworthy that the UPK29/33 fork was previously detected in tryptic digests of Ub-conjugates in growing yeast (27). Although real quantitation of these forks is not yet possible, it is clear that CHIP, MuRF1, Mdm2, and presumably other U-box and Ring finger E3s with UbcH5 as the E2 synthesize novel Ub chains that are composed of diverse types of linkages and bifurcations (Table 1 and Fig. 2C). Moreover, because these E3s with UbcH5 form three types of Ub forks (UPK6/11, UPK27/29, and UPK29/33), it seems likely that they also form forked chains with two Ubs linked to nonadjacent lysines on the proximal Ub (e.g. to Lys48 and Lys63). Thus, these novel chains may even be arborized and contain multiple forks in tree-like structures. However, the present approaches cannot test if two Ubs are linked to nonadjacent lysines on the proximal Ub, because the treatment with trypsin (or the other protease mixture) would have cleaved between these two lysines.

CHIP and MuRF1 Form Homogeneous Lys63 Chains When They Act with the E2, UbcH13/Uev1a—Additional experiments tested whether the formation of forked chains containing all linkages was related to the E2, UbcH5. Recently CHIP, with the dimeric E2, UbcH13/Uev1a, was reported to form free polyUb chains that were not linked to a substrate (9). We therefore tested whether CHIP may also ubiquitinate luciferase with this E2 (in place of UbcH5) together with E1, Hsp70, and ATP (supplemental Fig. 1A and B). As shown with its yeast homolog Ubc13/Mms2 (29), the UbcH13/Uev1a dimer by itself formed anchorless polyUb chains (supplemental Fig. 1A). With CHIP present, these anchorless Ub chains were formed more rapidly, but in contrast to prior reports, we found that Ub-protein conjugates were also synthesized. These conjugates corresponded to ubiquitinated luciferase, as shown by Western blot analysis (supplemental Fig. 1B). Thus a single E3 can catalyze ubiquitination of the same substrate (luciferase) with two different types of E2s (UbcH5 and UbcH13/Uev1a).

Because the UbcH13/Uev1a dimer alone forms anchorless Lys63 linkages, we tested various Ub mutants to test whether CHIP in conjunction with this E2 forms on the substrate Ub chains composed only of Lys63 linkages or whether they form heterogeneous forked chains resembling those CHIP forms with UbcH5 (Fig. 3, A and B). As expected, all the single-substitution Ub mutants with the exception of K63R supported the formation of anchorless Ub chains (Fig. 3A, 5th to 11th lanes). Similarly, luciferase was ubiquitinated by CHIP with all single-substitution Ub mutants except K63R (Fig. 3B, upper panel). The K63R mutant like methylated Ub could not form polyubiquitination of the same substrate (luciferase) with two different types of E2s (UbcH5 and UbcH13/Uev1a).
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ubiquitinated luciferase under these conditions. Furthermore, CHIP could not ubiquitinate luciferase using any single-lysine Ub, except Lys^63 (data not shown). With UbCH13/Uev1a, CHIP (Fig. 3B, middle panel), unlike with UbCH5, showed no autoubiquitination, as was also reported by Zhang et al. (9), or ubiquitination of Hsp70 (Fig. 3B, lower panel). Together these findings indicate that CHIP forms homogeneous chains on the substrate with UbCH13/Uev13, in sharp contrast to its mode of ubiquitination with UbCH5.

To test whether Ring finger E3s also form polyUb chains with UbCH13/Uev1a, we assayed ubiquitination by MuRF1. As noted previously, UbCH13/Uev1a formed anchorless polyUb chains without an E3. However, addition of MuRF1 enhanced strongly their synthesis (as found with CHIP) (supplemental Fig. 2A). With this dimeric E2, very little autoubiquitination of MuRF1 was seen, in contrast to the rapid autoubiquitination seen with UbCH5 (supplemental Fig. 2, B and C). We then investigated the type of Ub linkages formed by MuRF1 and UbCH13/Uev1a (Fig. 3C). Wild type Ub (Fig. 3, 2nd lane) and all single-substitution Ub mutants (4th to 9th lanes), except K63R (10th lane) or methylated Ub (3rd lane), supported formation of anchorless chains as reported previously. To test whether MuRF1 with UbCH13/Uev1a can also ubiquitinate a substrate through Lys^63 linkages, we assayed the ubiquitination of tropinin I using these various Ub mutants (Fig. 3, D and E). All single-substitution Ub mutants except K63R supported the ubiquitination of troponin I (Fig. 3D). Furthermore, when various single-lysine Ub mutants were used, only the Lys^63 single-lysine mutant supported troponin I ubiquitination (Fig. 3E). Thus, both the monomeric U-box and Ring finger E3s can catalyze protein ubiquitination by binding the substrate and enhancing the inherent capacity of UbCH13/Uev1a to form Lys^63 linkages.

MuRF1 with UbCH1 (E2–25K) Forms a Homogeneous Lys^48 Chain on Substrate—We then determined whether any other E2 also can support formation of forked, heterogeneous chains by MuRF1 (Fig. 4, A and B). As reported previously, UbCH1 and UbCH13/Uev1a by themselves form anchorless polyUb chains, and UbCH3 (mammalian Cdc34) ubiquitinates itself (Fig. 4A). Only UbCH1, UbCH5, and UbCH13/Uev1a formed large polyUb-conjugates in the presence of MuRF1. Surprisingly, MuRF1 also polyubiquitinated its substrate troponin I with UbCH1 (Fig. 4B, 2nd lane) as well as with UbCH5 (5th lane) and UbCH13/Uev1a (8th lane), and this modification of troponin I was not seen in the absence of MuRF1 (Fig. 4C). Unlike MuRF1, the U-box E3, CHIP, could not ubiquitinate luciferase with UbCH1 (data not shown). Interestingly, ubiquitination of troponin I by MuRF1 with UbCH1 or UbCH13/Uev1a was quite processive and produces large Ub-troponin I conjugates; however, under similar conditions, ubiquitination by MuRF1 and UbCH5 is distributive and produces only rather small conjugates.

Even though UbCH1 is one of best characterized E2s (30, 31), few E3s are known to function with it (32, 33). By itself UbCH1 forms anchorless Lys^48 Ub chains (30). We therefore determined the isopeptide linkages formed by MuRF1 and UbCH1 on troponin I (Fig. 4, D and E). Using the seven single-substitution Ub mutants, we found that Lys^48 is required for the ubiquitination of troponin I by MuRF1 and UbCH1 (Fig. 4D, 9th lane).

Also of the various single-lysine Ub mutants, only Lys^48 supported troponin I ubiquitination (Fig. 4E, 9th lane). Thus MuRF1 with UbCH1 synthesized only Lys^48 linkages. Together these findings indicate that the type of isopeptide linkages formed by Ring finger or U-box E3s is determined by the E2.

The HECT Domain E3s, E6AP and Nedd4, Make Heterogeneous Lys^48 and Lys^63 Chains, Respectively—Because the mechanism of the HECT domain family of E3s differs from that of the Ring finger or U-box domain E3s, we examined whether the
HECT domain E3, E6AP, like the Ring finger and U-box E3s (Fig. 1) with UbcH5 synthesizes polyUb chains that contain all possible types of linkages and are forked. The HECT domain E3s form a highly reactive thioester bond with the Ub before ligating it to the substrate (11). E6AP can form a long polyUb chain with wild type Ub present (Fig. 5A, 1st lane), but not with lysine-less (2nd lane) or methylated Ub (10th lane). Unlike the U-box or Ring finger ligases, E6AP did not form a polyUb chain with any single-lysine Ub mutant (Fig. 5A, 3rd to 9th lanes). This finding is not consistent with the prior report that E6AP can form polyUb chain with single-lysine Ub mutants (14). We therefore used single-substitution Ub mutants (Fig. 5B) to learn which isopeptide linkages were synthesized by E6AP. E6AP could not form a polyUb chain with K48R Ub, although it did so with the other Ub mutants to a similar extent as with wild type Ub (with the possible exception of K11R Ub). Thus E6AP seemed to form only Lys48 isopeptide linkages, as reported previously (14, 34).

To confirm these conclusions, the polyUb chains formed by E6AP during autoubiquitination of GST-E6AP with UbcH5 and wild type Ub were analyzed by mass spectrometry, which showed that they were composed almost exclusively of Lys48 linkages (Table 2). Even though K11R Ub supports the autoubiquitination of E6AP to a lesser extent than other single-lysine Ub mutants, UPK11 was not detected by mass spectrometry. To check whether E6AP during autoubiquitination forms similar linkages as during ubiquitination of a substrate protein, we analyzed the polyUb chain formed on HHR23A (expressed in *Escherichia coli*) by E6AP with UbcH5 (35) (Fig. 5C). The ubiquitinated HHR23A was found by mass spectrometry also to contain only Lys48 isopeptide linkages (Table 2). These findings together suggest that the formation of the Ub thioester on E6AP somehow restricts the type of isopeptide linkages formed.

To determine whether other HECT domain E3s behave like E6AP, we studied another member of this ligase family, Nedd4 (36), and analyzed the linkages it forms during autoubiquitination. Like E6AP, Nedd4 was efficient at autoubiquitination with wild type Ub, but it did not form a polyUb chain with any single-lysine Ub mutant (data not shown). Thus HECT domain E3s, unlike other families of E3s, seem to require multiple lysines on the Ub to form polyUb chains. However, Nedd4 formed a polyUb chain with all the single-substitution Ub mutants, with the clear exception of K63R (Fig. 5D). Interestingly, with K11R Ub, Nedd4 was less active in polyUb chain formation than with other such mutants, as was also found with E6AP. Analysis by

**TABLE 2**
Demonstration by mass spectrometry that Cdc34, SCF complex, and HECT domain E3s form homogeneous Ub chains containing specific linkages

| Ub peptide | Cdc34
textsuperscript{a} | Cdc34 + Cdc34
textsuperscript{b} | E6AP + UbcH5
textsuperscript{c} | Nedd4 + UbcH5
textsuperscript{d} |
|------------|-----------------|-----------------|-----------------|-----------------|
| Linear     |                 |                 |                 |                 |
| UPK6       | 0               | 0               | 0               | 0               |
| UPK11      | +               | +               | 0               | 0               |
| UPK48      | + + + +         | + + + +         | + + +           | + + +           |
| UPK63      | +               | 0               | 0               | 0               |
| Forked     |                 |                 |                 |                 |
| UPK6/11    | 0               | 0               | 0               | 0               |
| UPK27/29   | 0               | 0               | 0               | 0               |
| UPK29/33   | 0               | 0               | 0               | 0               |


textsuperscript{a} Enzyme.

textsuperscript{b} Substrate.
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mass spectrometry confirmed that the polyUb chain formed by Nedd4 is composed only of Lys63 linkages (Table 2). Thus the HECT domain E3s have the capacity to make absolutely homogeneous Ub chains, but the nature of the chain can vary with the E3. They generate chains composed purely of Lys48 linkages with E6AP or Lys63 linkages with Nedd4 with UbcH5, while the Ring finger or U-box E3s with UbcH5 form mixed linkages and even forked chains. Thus the nature of both the E2 and the E3 can determine the nature of the chains formed.

26S Proteasomes Rapidly Degrade Troponin I Linked to Lys48 or Lys63 but Not Mixed Forked Chains—These findings enabled us to test whether these mixed forked chains and the homogeneous Lys48 and Lys63 chains can support degradation by pure 26S proteasomes. It is widely stated that only polyUb chains composed of Lys48 or Lys29 linkages to proteins mediate the proteasomal degradation of the proteins (12, 13, 15), and that Lys63 chains function in DNA repair or certain signaling cascades, although proteasomal degradation of dihydrololate reductase chemically linked to the Lys63-linked tetra-Ub chain were reported previously (29). The discovery that the same E3, MuRF1, with different E2s can form Lys48, Lys63, or mixed forked chains on troponin I enabled us to compare the capacity of these different chains to support degradation by pure 26S proteasomes. Troponin I was ubiquitinated by MuRF1 with UbcH1, UbcH5, or UbcH13/Uev1a, and degradation of troponin I was assayed by addition of purified rabbit 26S proteasomes to the reaction mixture.

As expected, troponin I ubiquitinated by MuRF1 and UbcH1 through Lys48 linkages was degraded rapidly (Fig. 6B). By contrast, in the absence of ubiquitin there was no degradation of troponin I. Surprisingly, troponin I ubiquitinated through Lys63 linkages with UbcH13/Uev1a was also degraded efficiently by purified proteasomes and, in fact, was degraded consistently more rapidly than when it was ubiquitinated through Lys48 linkages (Fig. 6B). This finding clearly does not support the widespread conclusion that Lys63 chains do not support proteasomal degradation (3). By contrast, the forked Ub chain containing all types of isopeptide linkages formed with UbcH5 did not support significant degradation of troponin I. The resistance of these mixed forked chains to proteasomal degradation is noteworthy because UbcH5 is abundant and has often been implicated in proteolysis in vivo. A similar failure of degradation was seen in related experiments when luciferase was linked to mixed forked Ub chains using CHIP/UbcH5. In fact, with luciferase and CHIP (and Hsp70) as the E3, less than 1% of the substrate was degraded by pure proteasomes (data not shown). Moreover, in addition to the very poor degradation of troponin I and luciferase, the mixed forked Ub chains formed with UbcH5 remained largely intact despite the presence of 26S proteasomes in sharp contrast to the Lys48 and Lys63 chains that were lost concomitantly with the ubiquitinated substrate (Fig. 6A and data not shown). Specifically, 70% of the mixed forked conjugates were still in high molecular weight form despite the presence of 26S proteasomes, but only 30% of the Lys48 chains and only 5% of the Lys63 Ub chains. During incubation with the 26S proteasomes, the amount of ubiquitinated troponin I containing Lys48 (because of the presence of UbcH1) or Lys63 chains (because of UbcH13/Uev1a) decreased. However, there was no reduction

FIGURE 6. Forked Lys48/Lys63 isopeptide linkages are more resistant to deubiquitination by purified 26S proteasomes than standard Ub linkages. A, degradation of troponin I ubiquitinated by MuRF1 and UbcH1, UbcH5, or UbcH13/Uev1a was measured using purified 26S proteasomes. Although troponin I ubiquitinated with UbcH1 (through Lys48 linkages) was degraded rapidly, troponin I ubiquitinated with UbcH5 was degraded only slowly. Interestingly, the Lys63 chains supported the degradation of troponin I very efficiently. The same blot was overexposed to measure disassembly by proteasomes of each chain longer than 4 Ub (upper panel, marked by asterisk). In the presence of the proteasomes, only 30% of the Lys48 and 5% of the Lys63 chains present initially (i.e. in the reactions lacking proteasomes) were in high molecular weight form. However, 70% of the mixed forked chains were not disassembled. Troponin I was detected by Western blotting with anti-troponin I antibody. This result was a typical of findings obtained in three independent experiments. B, proteasomal degradation of Ub-troponin I conjugates containing a different type of isopeptide linkages was calculated by measuring the remaining troponin I with a densitometer. Although troponin I linked to Lys48 or Lys63 chain was degraded rapidly (34 and 47% each in an 1-h reaction), less than 2% of troponin I linked to mixed forked Ub chain was degraded. These data are the average of three independent experiments. C, effects of incubation of Ub-conjugates with proteasomes. After incubation with 26S proteasomes, fewer high molecular weight conjugates were found on SDS-PAGE. Luciferase was ubiquitinated by CHIP with wild type Ub and then isolated with an anti-luciferase antibody and protein A/G resin. Luciferase bound to the resin was incubated at 37 °C for 1 h with buffer (1st lane) or with 0.4 μg of 26S proteasomes (2nd lane). Luciferase was detected by Western blotting with an anti-luciferase antibody. This gel is typical of results obtained in three independent experiments. D, Western blotting with an anti-luciferase antibody. This gel is typical of results obtained in three independent experiments. E, Western blotting with an anti-luciferase antibody. This gel is typical of results obtained in three independent experiments.
in troponin I linked to mixed forked chains (formed with UbcH5). These findings indicate a resistance of these novel chains to proteasomal isopeptidases or a failure to bind correctly to the 26S particle.

Forked Linkages Are Relatively Resistant to Isopeptidases on 26S Proteasomes—As noted, the mixed forked ubiquitin chains both failed to support protein degradation and also appeared relatively resistant to disassembly by the 26S proteasome-associated isopeptidases (Fig. 6A). This resistance of mixed forked Ub chains to proteolysis and disassembly by the 26S particles could be due either to the presence of the heterogeneous isopeptide linkages or to the forked chain, either of which might decrease conjugate binding to the 26S proteasome or make it less susceptible to the three deubiquitinating enzymes associated with mammalian 26S proteasomes, UBP6 (37, 38), UCH37 (39), and Rpn11 (40, 41). To compare the susceptibility of the forked and standard isopeptide linkages to the proteasome-associated isopeptidases, luciferase was ubiquitinated by CHIP and UbcH5, and the Ub-conjugates were isolated with an antiluciferase antibody. The Ub-conjugates were then incubated with purified 26S proteasomes and ATP for 1 h, and the residual polyUb-luciferase conjugates were then resolved on SDS-PAGE. After incubation with the proteasomes, the amount of longer Ub-conjugates decreased (Fig. 6C).

To compare the sensitivity of different Ub linkages to proteasomal isopeptidases, the high molecular weight Ub-luciferase conjugates (larger than the 188-kDa marker) were recovered from the gel and digested with trypsin. The resulting peptides were analyzed by nano-LC-MSMS, and the quantities of each UPK were determined by comparison with an internal standard (isotopically labeled UPK48, UPK48H), using the SIM method (Fig. 6D). The changes in other UPKs were calculated by comparison of the levels of each in the three preparations after normalization with the same amount of the internal standard, UPK48H. During incubation for 1 h with 26S proteasomes, the quantity of UPK48 decreased by 74% (i.e. from 537 to 138 fmol measured by using the internal standard), UPK11 decreased by 61%, and UPK63 by 76% (Fig. 6E). By contrast, the quantity of the forked peptide UPK6/11 fell by only 28%, and as a result, the relative content of UPK6/11 increased at least 2–3-fold following incubation. As noted above, we could not test whether UPK27/29 or UPK29/33 was also enriched because of the inability to quantitate them reliably. Nevertheless, it is clear that the 26S proteasomes disassembled the forked Ub chains less efficiently than the other types of Ub linkages. Presumably, this resistance of the forked chain to the proteasomal isopeptidases can explain (at least in part) why troponin I or luciferase linked to these forked Ub chains are degraded very poorly.

This resistance of the mixed forked chains to proteasomal degradation and chain disassembly may also be because of a failure to bind tightly to the 26S proteasome. To test this possibility, we examined whether these novel structures could inhibit the degradation of other Ub-conjugates. We measured proteasomal degradation of troponin I ubiquitinated by MuRF1 and UbcH13/Uev1a (i.e., Lys53 chains) in the presence of pre-synthesized mixed forked Ub-luciferase conjugates (formed by CHIP and UbcH5) or Lys53 Ub-luciferase conjugates (formed by CHIP and UbcH13/Uev1a). Although luciferase linked to a Lys63 chain could inhibit degradation of troponin I, presumably by competition for binding to the 26S, luciferase linked to a mixed forked chain failed to inhibit the breakdown of troponin I in several experiments (data not shown). These findings suggest that these novel structures have a lower affinity for 26S proteasome than do the homogeneous chains.

DISCUSSION

Formation of Homogeneous or Heterogeneous Forked Ub Chains—The present findings call into question many widespread generalizations concerning the ubiquitin proteasome pathway. Specifically, it has been generally assumed that each E3–E2 pair synthesizes Ub chains composed of a single type of isopeptide linkage, that polyUb chains composed of different types of linkages confer distinct fates on the protein, and that Lys63 chains do not support proteasomal degradation (3). As shown here, the HECT domain E3s, E6AP and Nedd4, and the U-box E3 CHIP or Ring finger E3, MuRF1, with UbcH13/Uev1a or UbcH1 (E2–25K) do in fact generate chains composed exclusively of a single kind of linkage. Also, the SCF complex with Cdc34 or Cdc34 by itself generates chains containing predominantly (but not exclusively) a single kind of linkage (Table 2). In contrast, several physiologically important E3s, the U-box E3, CHIP, and the Ring finger E3s, MuRF1 and Mdm2, with UbcH5 form polyUb chains containing all seven possible isopeptide linkages. It is noteworthy that in the polyUb chains formed by CHIP, MuRF1 and Mdm2 with UbcH5, the most abundant linkages were through Lys11, Lys48, and Lys63 and that in the polyUb chains found in growing yeast, the same linkages are also more abundant than other linkages (27), although in in vivo studies these linkages were assumed to be in distinct chains. It is also noteworthy that with no E2–E3 pair studied here was evidence found for N-terminal ubiquitination.

Even more surprising was the finding that these polyUb chains contain “forks,” in which two Ub moieties are linked to adjacent lysine residues at Lys6 + Lys11, Lys27 + Lys29, or Lys29 + Lys33 on the proximal Ub (Table 1 and Fig. 2, B and C). These heterogeneous, bifurcated chains were synthesized under typical conditions commonly used in such studies (e.g. 1-h incubations with typical concentrations ratios of E3, E2, and substrate), and therefore they must have been formed, but not recognized, in prior studies. For the detection of forks, we chose the condition where the ubiquitination process is still linear (1 h) and only a fraction of the substrate was consumed. Therefore, the forks are not formed only under extreme conditions when no other substrate is available. Furthermore, the nature of the linkages seemed similar in short chains (5–14 Ubs) and longer chains (>14 Ubs) (data not shown). Similar findings about the composition of the conjugates were obtained using mass spectrometry and in experiments with the various Ub mutants. This agreement strongly supports the validity of both approaches, but only mass spectrometry could reveal the existence of the forked chains and the relative amounts of linkages in the heterogeneous chains.

Our prior study had reported that the chains found in growing yeast contained forked linkages at Lys29 and Lys33 (27). That study did not describe the Lys6 + Lys11 or Lys27 + Lys29 forks reported here, but these structures would have been very diffic-
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cult to demonstrate in cells because the conjugates isolated from cells are highly heterogeneous, and the UKPs are mixed with an enormous number of tryptic peptides derived from diverse ubiquitinated substrates. In any case, these observations suggest that a significant number of E3s in vivo function in a similar fashion as found here for the pure monomeric E3s, CHIP, MuRF1, and Mdm2 with UbcH5. The discovery of these heterogeneous Ub chains raises the fundamental question of how these mixed forked chains are formed by certain E3s, whereas other families of E3s generate chains composed predominantly or exclusively of Lys\(^{48}\) or Lys\(^{63}\) linkages.

During the course of these studies, Kirkpatrick et al. (42) reported that the very large multisubunit E3 APC/C forms with Ubc10 or Ubc4 on cyclin B1 another novel type of Ub chain composed of mixed isopeptide linkages. Like the chains formed by the small Ring finger and U-box E3s (Table 1), those formed by APC/C, which contains a catalytic Ring finger subunit, were composed primarily of Lys\(^{48}\), Lys\(^{63}\), and Lys\(^{11}\) linkages plus some Lys\(^{6}\) and Lys\(^{55}\) linkages. Interestingly, no forked linkages were observed in the analysis of the chains formed by APC/C on cyclin B1. This difference might be a consequence of the different E2s or because the multisubunit APC/C complex has a special mechanism to prevent the formation of forked chains.

The fact that the forks were present in chains containing all possible Ub linkages strongly suggests that the formation of an isopeptide linkage by these E3s with UbcH5 is a rather random process, which occasionally links two Ub molecules to the proximal ubiquitin. Therefore, it is likely that Ub forks are formed even more often through the linkage of two Ub molecules to nonadjacent lysines on the preceding Ub, especially to Lys\(^{11}\) + Lys\(^{48}\) or Lys\(^{48}\) + Lys\(^{63}\), because these are the most abundant sites of isopeptide linkages. Peptides indicative of such structures cannot be generated by treatment with trypsin (which cleaves at the intervening lysines) or by use of the alternative protease mixture and therefore cannot be detected by mass spectrometry. If such distant forks are also formed by these Ring finger and U-box E3s, as seems very likely, then the Ub chains formed by these E3s may be highly forked or “arborized.”

UbcH5, but Not Other E2s, Forms Mixed Forked Ub Chain with Ring Finger and U-box E3s—As these studies clearly indicate, the nature of both the E3 and the E2 are important in determining the type of isopeptide linkages formed. In the absence of an E3, certain E2s catalyze the formation of polyUb chains containing only a single type of linkage, e.g. UbcH1 (E2–25K) (30), and Cdc34 forms Lys\(^{48}\) linkages (43) as confirmed here, whereas E2EPF forms only Lys\(^{11}\) and Rad6 only Lys\(^{6}\) linkages (44). By mass spectrometry, the Lys\(^{48}\) chains formed by autoubiquitination of Cdc34 were highly uniform in composition with only trace amounts of Lys\(^{11}\) and Lys\(^{55}\) linkages (Table 2). The polyUb chains formed on Sc1c1 by the SCF with Cdc34 also contained overwhelmingly Lys\(^{48}\) linkages (with a small amount of Lys\(^{11}\) bonds), which presumably explains why Sc1c1 ubiquitinated in the presence of Cdc34 is rapidly degraded in vitro (45, 46). During ubiquitination of a substrate, the Ring finger subunit of SCF, Rbx1, releases the activated Cdc34-Ub prior to transfer of the Ub to the growing chain (47); this feature can explain why SCF on the substrate forms similar types of chains as Cdc34 forms alone (Table 2).

By contrast, the small monomeric U-box or Ring finger E3s, which are the most abundant types of E3s in the genome, appear to catalyze the formation of mixed polyUb chains by recruiting the substrate and Ub-UbcH5 and simply releasing the highly reactive Ub (10) or E2-Ub (47) in the vicinity of the substrate. Such a mechanism could account for what appears to be the nonspecific transfer by Mdm2, MuRF1, and CHIP of Ub from UbcH5 to any free NH\(_2\) group on the preceding Ub (Fig. 1 and Table 1) and the formation of forked Ub chains (see below). Because these findings were obtained with all monomeric Ring finger or U-box E3s of this type investigated, formation of heterogeneous forked chains probably reflects the behavior of this large family of enzymes in the presence of UbcH5 (although the dimeric Ring finger E3 Brca1-Bard1 has been reported with UbcH5 to form chains containing primarily Lys\(^{6}\) linkages) (14, 16).

This nonspecific pattern of ubiquitination was only found when these monomeric E3s functioned with UbcH5. Although we confirmed the recent report that CHIP can catalyze formation of anchorless Lys\(^{63}\)-specific polyUb chains with UbcH13/Uev1 (9), which by itself mediates the formation of such chains (48), we found that CHIP with UbcH13/Uev1a can also ubiquitinate a protein substrate with chains composed only of Lys\(^{63}\) linkages (Fig. 3). Furthermore, MuRF1 was shown to behave similarly with UbcH13/Uev1 and also to form exclusively Lys\(^{63}\)-linked chains that are either anchorless or linked to its substrate, troponin I (Fig. 3). Thus, a single E3 acting on a substrate can form distinct linkages using two different E2s.

With the yeast homolog of UbcH13/Uev1a, Ubc13/Mms2, this formation of Lys\(^{63}\) linkages has been shown to be due to the noncovalent binding on Mms2 of the preceding Ub so as to transfer the Ub from the E2 specifically to Lys\(^{63}\) (49, 50). In this case, as in SCF working with Cdc34 (51), the E3 appears to accelerate on the substrate the same process of chain synthesis, which the E2 can slowly catalyze by itself. More interestingly, we found that MuRF1 with UbcH1 ubiquitiniates troponin I through only Lys\(^{48}\) linkages (Fig. 4). It has been reported that the C-terminal UBA domain of UbcH1 is required for polyUb chain synthesis (31, 52), and if their UBA domain is deleted, UbcH1 forms irregular Ub chains (53). Presumably, this Ub-binding domain orients the Ub and determines the type of isopeptide linkage formed (in a similar fashion as Mms2 with Ubc13 (49)).

Unlike MuRF1 and Mdm2, SCF is composed of multiple subunits, including the substrate recognition subunit (F-box protein), as well as Cullin, and the Ring finger protein Rbx1 that is essential for chain formation (54). Possibly, this architecture of the SCF complex helps ensure that during ubiquitination, the preceding ubiquitin, and the incoming Ub–E2 thioester are maintained in a specific orientation to yield homogeneous Lys\(^{48}\) chains. However, the E2, Cdc34, appears critical in determining that Lys\(^{48}\) chains are formed. In fact, non-Lys\(^{48}\) isopeptide linkages are formed when the core proteins of the SCF complex function with UbcH5 instead of Cdc34 (55). Also, Petroski and Deshaies (51) have demonstrated a key feature of Cdc34 that is absent in UbcH5, an acidic loop of Cdc34 that seems to ensure the formation of Lys\(^{48}\) linkages. Recently, both Cdc34-Ub and UbcH5-Ub have been reported to be activated.
through the formation of a noncovalent complex (54, 56), which also appears to contribute to the processing of ubiquitination and perhaps the nature of the linkage synthesized.

In contrast, with the HECT domain group of E3s, it is the E3 and not the E2 that determines the type of isopeptide linkages, because Nedd4 and E6AP with UbcH5 formed different types of homogeneous chains. When E6AP catalyzed autoubiquitination with UbcH5 or acted on the substrate, HHR23A, the polyUb chains contain only Lys$^{48}$ linkages (Table 2). However, Nedd4 with UbcH5 during autoubiquitination formed chains containing only Lys$^{63}$ linkages. Interestingly, unlike the two HECT domain E3s studied here, another family member, KIAA10, has been reported to synthesize anchorless Ub chains containing Lys$^{29}$ and Lys$^{48}$ isopeptide linkages (57). The exact structure of these enzymes must limit which of the seven lysine residues of ubiquitin are able to carry out a nucleophile attack on the E6AP-Ub thioester, and must position precisely the proximal Ub in a similar manner as the acidic loop in Cdc34 or the loosely bound preceding Ub in Ubc13/Uev1. Clearly, CHIP and the monomeric Ring finger E3s lack such a positioning mechanism when functioning with UbcH5 and presumably with any of the related monomeric E2s.

**Proteasomal Processing of Lys$^{48}$, Lys$^{63}$, and Mixed Forked Chains**—The present findings of novel polyUb chains that contain all possible linkages and some (perhaps multiple) bifurcations raise many obvious questions concerning the possible biochemical and biological significance of these novel structures. The most obvious question is whether such novel structures can support degradation by proteasomes. We found that troponin I linked to the forked mixed Ub chain by MuRF1 with UbcH5 was degraded quite poorly, if at all, by purified 26S proteasomes. In contrast, troponin I linked to the homogeneous Lys$^{48}$ or Lys$^{63}$ chains was rapidly digested. In related studies, we have found that luciferase linked to such mixed forked chains also resists proteasomal degradation. This resistance to degradation of the mixed forked chain seems to account for the frequent failure of investigators to reconstitute efficient protein degradation with pure Ring finger E3s and proteasomes. Presumably in vivo, these mixed forked chains serve another function or there are additional factors that ensure their rapid degradation.

Another important and quite surprising finding was that when troponin I was linked to Lys$^{63}$ chains by MuRF1 and UbcH13/Uev1, it was rapidly degraded. In fact, the Lys$^{63}$ chains appeared to consistently support more rapid degradation of this substrate by pure 26S proteasomes than Lys$^{48}$ chains (formed by MuRF1 with UbcH1). In accord with these findings, Hofmann and Pickart (29) had initially noted that synthetic Lys$^{63}$-linked tetra-Ub chains can support degradation by purified proteasomes, although this observation has been generally ignored because subsequent in vivo work showed that Lys$^{63}$ chains on certain proteins play critical roles in signal transduction and DNA repair, rather than rapid degradation (3). The most likely explanation of this apparent contradiction may be that in cells or in specific cell compartments additional factors bind to the Lys$^{63}$-linked Ub protein conjugates and prevent their rapid degradation. However, the present findings indicate that even if Lys$^{63}$ chains on certain proteins can serve signaling functions, they may also eventually trigger proteosomal degradation of these proteins.

The resistance to degradation by pure proteasomes of the troponin I linked to mixed forked Ub-conjugates may be due either to the presence of the diverse types of isopeptide bonds or to the multiple forks. Normally, degradation by the proteasome and Ub chain disassembly occur concomitantly as seen in Fig. 6A with Lys$^{48}$ and Lys$^{63}$ chains. However, the mixed forked chains were much more resistant to disassembly by the 26S. It is also noteworthy that during incubation of these chains with the proteasome, the forked Ub linkages were more resistant than other isopeptide linkages to isopeptidases associated with the 26S proteasome (Fig. 6). Possibly these isopeptidases are unable to cleave the adjacent isopeptide linkages on the same Ub or they have less affinity for forked chains. One observation suggesting a lower affinity of such chains for the proteasome was our finding that homogeneous Lys$^{63}$-ubiquitinated luciferase could inhibit degradation of ubiquitinated troponin I, whereas the mixed forked Ub-luciferase conjugates could not. In either case, this resistance of the forks to disassembly (perhaps combined with decreased affinity of such conjugates for the 26S) may explain why proteins linked to mixed forked Ub chains are degraded very poorly. Accordingly, the mixed conjugates found on cyclin B by APC/C that are not forked are efficiently degraded by yeast 26S proteasomes (42). Although we cannot quantitate the abundance of these forks (and cannot even assay forks if they are to nonadjacent lysines), the resistance of these conjugates to proteasomal degradation suggests that forks are present in sufficient amounts to have major biochemical consequences.

It is well established in vivo that Mdm2 targets p53 for proteasomal degradation (58), that MuRF1 is essential for the rapid protein degradation associated with muscle atrophy (24), and that CHIP targets many proteins important in human disease for degradation, including cystic fibrosis transmembrane regulator, polylamine proteins, and hyperphosphorylated Tau (21, 22, 59). Because these Ring finger and U-box E3s with UbcH5 catalyze rapid degradation of specific proteins in vivo, cells probably contain factors that either prevent the formation of forked chains or facilitate their degradation. Recently, we have found that intracellular Ub-binding proteins can alter the ubiquitination process by the Ring finger and U-box E3s with UbcH5, so as to reduce the formation of forked chains. They thus ensure the production of chains that are degradable by 26S proteasomes.

However, because these novel mixed or forked chains are readily formed by physiologically important ubiquitination enzymes, they may serve some specific functions in vivo, aside from degradation, or have distinctive properties that are important in cell regulation. Alternatively, these novel chains may in vivo only be occasional untoward (“off-pathway”) consequence of misubiquitination but may be important in pathological states, e.g. in neurodegenerative disease, where Ub-conjugates resist degradation and disassembly (60).

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5 H. T. Kim, K. P. Kim, S. P. Gygi, and A. L. Goldberg, manuscript in preparation.
Formation of Forked Polyubiquitin Chain

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