Protein modification with lysine 63-linked ubiquitin chains has been implicated in the non-proteolytic regulation of signaling pathways. To understand the molecular mechanisms underlying this process, we have developed an in vitro system to examine the activity of the ubiquitin-conjugating enzyme UBC13-UEV1A with TRAF6 in which TRAF6 serves as both a ubiquitin ligase and substrate for modification. Although TRAF6 potently stimulates the activity of UBC13-UEV1A to synthesize ubiquitin chains, it is not appreciably ubiquitinated. We have determined that the presentation of Lys63 of ubiquitin by UEV1A suppresses TRAF6 modification. Based on our observations, we propose that the modification of proteins with Lys63-linked ubiquitin chains occurs through a UEV1A-independent substrate modification and UEV1A-dependent Lys63-linked ubiquitin chain synthesis mechanism.

The post-translational modification of proteins through the covalent addition of the 76-amino acid protein ubiquitin has emerged as a critical mechanism for regulating many aspects of cellular physiology (1). The role of ubiquitin in modulating protein activity is defined in part by the extent and type of modification. Single ubiquitin molecule attachments (mono-ubiquitination) as well as chains of ubiquitin molecules generated through isopeptide linkages between the primary amine of a lysine side chain of one molecule and the C terminus of another encode signals that are deciphered to trigger distinct cellular responses.

Chains of specific ubiquitin-ubiquitin linkages result in distinct protein fates. For example, a lysine 48-linked chain of at least four ubiquitin molecules is the minimum signal for efficient degradation of a protein by the 26 S proteasome in vitro (2). Emerging evidence suggests, in contrast, that Lys63-linked ubiquitin chains attached to a protein serve in non-proteolytic functions such as regulating intracellular signaling (3, 4). Other types of ubiquitin chains also exist including those containing mixed linkages and branched chains (5, 6). However, these have been primarily observed as products of in vitro reactions and their physiological significance is unclear.

Perhaps the most well understood mechanism for generating specific ubiquitin linkages is through the activity of the ubiquitin-conjugating enzyme (E2)2 UBC13. UBC13 functions as a heterodimer by binding through a large hydrophobic interface with the UEV (ubiquitin-conjugating enzyme variant) family of which MMS2 and UEV1A are representative members (4, 7). UEVs contain the conserved enzymatic core of ubiquitin-conjugating enzymes, but lack a catalytic cysteine. By binding a ubiquitin molecule and positioning its Lys63 near the active site cysteine of UBC13, UEVs promote Lys63-linked ubiquitin chain synthesis (8).

Structural and biophysical studies have uncovered the molecular basis of how UBC13-UEV generates Lys63-linked ubiquitin chains (4, 7–12), yet little is known about how this ubiquitin-conjugating enzyme functions with its cognate ubiquitin ligases to modify protein substrates. To study this process, we have focused on reconstituting the modification of TRAF6 by UBC13-UEV1A in vitro with purified proteins. TRAF6 functions downstream of specific members of the tumor necrosis factor receptor family and upon receptor stimulation promotes NF-κB activation (13, 14). Fractionation experiments initially identified UBC13 as a TRAF6 interacting protein and a variety of cell-based and in vitro experiments suggest that TRAF6 modification with Lys63-linked ubiquitin chains has a non-proteolytic function in NF-κB activation through the kinase TAK1 (15–17). In this case, TRAF6 appears to function both as a ubiquitin ligase (it contains a RING motif and interacts with its ubiquitin-conjugating enzyme UBC13-UEV1A) and substrate for its own modification (it is subjected to “auto-ubiquitination”). Here, we examine how UBC13-UEV1A functions with this ubiquitin ligase to promote TRAF6 auto-ubiquitination and uncover that the activity provided by UEV1A primarily promotes Lys63-linked ubiquitin chain synthesis that effectively reduces TRAF6 modification. Based on these observations, we propose a model for how substrate modification with Lys63-linked ubiquitin chains occurs through the activity of this ubiquitin-conjugating enzyme.
Ubiquitination Reactions

Reactions were performed in ubiquitination reaction buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1 mM ATP, and 0.5 mM dithiothreitol. The indicated proteins were assembled on ice and the reaction was initiated by the addition of TRAF6 (where added) prior to incubation for 30 min at 37 °C. Reactions (10 µl) contained the following protein concentrations: 150 nM E1, 156 µM ubiquitin or various ubiquitin derivatives, 5 µM UBC13, 5 µM UVE1A, 5 µM UbcH5c, and 300 nM TRAF6. Under these conditions, TRAF6 is limiting relative to E2 based on our titration experiments examining ubiquitin discharge from UBC13 (Fig. 7). For reactions lacking a particular reaction component as indicated, an equivalent volume of ubiquitination reaction buffer was added. At the end of the incubation, an equal volume of denaturing sample buffer was added and the reactions were heated to 70 °C for 5 min prior to analysis by SDS-PAGE and immunoblotting with anti-ubiquitin (P4D1, Santa Cruz Biotechnology) and anti-TRAF6 (H-274, Santa Cruz Biotechnology) antisera. For time course experiments (Fig. 4), 10-µl aliquots of a 70-µl reaction (conditions as described above) were removed at the indicated times, added to reducing sample buffer, heated to 70 °C for 5 min and analyzed with anti-ubiquitin and anti-TRAF6 immunoblotting.

To analyze TRAF6 modification (Fig. 5), TRAF6 was bound to streptavidin-Sepharose (10-µl bead volume with 250 µl of TRAF6 baculovirus-infected insect cell lysate). After washing the Sepharose 3 times with Lysis Buffer (described above) and 2 times with ubiquitination reaction buffer, the indicated E2 subunits (5 µM) were added to reactions containing a final concentration of 150 nM E1 and 100 µM ubiquitin in a 20-µl total volume. After 20 min at room temperature with mixing, non-reducing sample buffer was added directly to 1 set of reactions ("soluble" reactions). The Sepharose for the other set of reactions ("bound" reactions) was washed in Lysis Buffer. TRAF6 was subsequently eluted from the streptavidin-Sepharose by a volume of non-reducing sample buffer equivalent to the total volume of the soluble reactions. Soluble and bound reactions were analyzed by SDS-PAGE and immunoblotting with anti-ubiquitin and anti-caldesmon-binding peptide (Upstate).

UBC13-Ub Thioester Discharge Assays

UBC13 (2 µM final concentration in 10 µl of charging reaction) was added to reactions containing ubiquitin (156 µM) and 150 nM E1 and incubated for 20 min at room temperature in ubiquitination reaction buffer (described above). The charging reaction was terminated by the addition of N-ethylmaleimide (NEM) (5 mM final concentration) and EDTA (10 mM final concentration), followed by a 15-min incubation at room temperature. Discharge reactions containing the various indicated proteins and ubiquitin (312 µM in final discharge reaction) were set up on ice in a ubiquitin reaction buffer without ATP. Reactions were initiated by the addition of an equal volume of charged and treated UBC13 (20 µl total volume), rapidly mixed, and incubated at 37 °C. Aliquots were removed at the indicated times and added to an equal volume of non-reducing SDS-PAGE sample buffer. UBC13-Ub thioester, UBC13, and TRAF6 were analyzed by SDS-PAGE and immunoblotting with anti-
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FIGURE 1. TRAF6 stimulates ubiquitin chain synthesis by UBC13-UEV1A. A, recombinant proteins were expressed, purified, and analyzed by SDS-PAGE and Coomassie staining. Approximately 1 \( \mu \)g of each protein was loaded for analysis. Ubiquitin-activating enzyme (E1, lane 2) was expressed from baculovirus-infected insect cells. UBC13 (lane 3) and UVE1A (lane 4) were expressed as individual subunits in E. coli and mixed to equimolar amounts. TRAF6 (lane 3) was expressed from baculovirus-infected insect cells. The relative positions of the various proteins are indicated. In vitro ubiquitination reactions were performed in the presence of the indicated proteins. After 30 min at 37 \( ^{\circ} \)C, reactions were stopped by the addition of denaturing SDS-PAGE sample buffer and analyzed for ubiquitin conjugation by anti-ubiquitin antisera (C). Free ubiquitin (Ub), ubiquitin chains, and TRAF6 are indicated.

UBC13 (Invitrogen) and anti-TRAF6 (Santa Cruz Biotechnology) antisera. The final concentrations of the proteins used in the reactions shown in Fig. 6 were 1 \( \mu \)M UBC13, 300 nM TRAF6, and 300 nM UEV1A. Fig. 7 utilized UBC13 at 500 nM and variable concentrations of either UVE1A or TRAF6, 200 nM, 500 nM, 1.25 mM, or 2.5 mM, to give the indicated molar ratios.

To examine potential downstream effects of NEM in chase reactions, UBC13 (2 \( \mu \)M) was charged with 3.75 \( \mu \)M ubiquitin containing an N-terminal FLAG epitope (Fig. 6, C and D). Reactions were either untreated (−NEM) or treated with NEM (+NEM, 5 \( \mu \)M final concentration) and EDTA (10 \( \mu \)M final concentration) for 15 min prior to adding to chase reactions containing 300 nM UEV1A and 300 nM TRAF6 and ubiquitin (312 \( \mu \)M). The final concentration of UBC13 in the chase reaction was 1 \( \mu \)M. Reaction aliquots were removed at the indicated times and analyzed under non-reducing conditions by immunoblotting with anti-FLAG horseradish peroxidase (Sigma) to detect UBC13-Ub thioester.

Molecular Modeling

The x-ray structures for UBC13-MMS2 (Protein Data Bank 1JAT) and MMS2-ubiquitin (PDB 1ZGU) were structurally superimposed on MMS2 using PyMol (Delano Scientific, San Carlos, CA) to generate a model of UBC13-MMS2-ubiquitin (root mean square 0.817). The distance from the amino group of the lysine 63 side chain to the sulfur of the active site cysteine of UBC13 was estimated from this model (see Fig. 8B).

RESULTS

Ubiquitin Chain Synthesis by UBC13-UEV1A Is Stimulated by TRAF6—The modification of TRAF6 with Lys\textsuperscript{63}-linked ubiquitin chains by UBC13-UEV1A promotes the activation of the kinase TAK1, which leads to the activation of NF-\( \kappa \)B gene expression (15, 16). Unlike ubiquitin ligases that recognize specific substrates that are targets for ubiquitin modification, TRAF6 functions both as a ubiquitin ligase and its own substrate. To study the molecular mechanisms underlying this process, we sought to recapitulate TRAF6 modification with Lys\textsuperscript{63}-linked ubiquitin chains in vitro using purified recombinant proteins (Fig. 1A).

We performed experiments to compare the activity of UBC13-UEV1A and TRAF6 separately to the complete reaction. Reaction products were subsequently analyzed for ubiquitin chain synthesis with an anti-ubiquitin antibody (Fig. 1B) and TRAF6 modification with anti-TRAF6 (Fig. 1C). Whereas UBC13-UEV1A synthesizes detectable amounts of ubiquitin chains after 30 min in the absence of a ubiquitin ligase, the addition of TRAF6 ligase, the addition of TRAF6 robustly stimulates this activity (Fig. 1B, compare lanes 1 and 3). As expected, TRAF6 had no detectable activity in the presence of E1, ATP, and ubiquitin (Fig. 1B, lane 2).

Despite TRAF6 efficiently stimulating the activity of UBC13-UEV1A to synthesize ubiquitin chains, we did not observe TRAF6 ubiquitination in comparing reactions performed with TRAF6 alone to reactions containing TRAF6 and UBC13-UEV1A (Fig. 1C, compare lanes 2 and 3). To rule out the possibility that our antibody cannot efficiently recognize ubiquitinated TRAF6 (generated against amino acids 1–274), we analyzed TRAF6 in these reactions with other antibodies generated against TRAF6 as well as one of the epitope tags present on this form of TRAF6 (calmodulin binding peptide) and obtained similar results (data not shown).

A Promiscuous E2, UbcH5c, Efficiently Ubiquitates TRAF6—Our observation that TRAF6 is not efficiently ubiquitinated in our in vitro system suggests that TRAF6 may not properly present an appropriate nucleophile (i.e. the amino group of a lysine residue side chain) for modification. Several recent reports have proposed that TRAF6 must oligomerize to form an active ubiquitin ligase complex and various proteins downstream of specific classes of tumor necrosis factor receptors (TIFA, MALT1, BCL10, and others) may facilitate this process (16, 18, 19). Thus, it is possible the deficiency of TRAF6 ubiquitination in our in vitro system may simply arise from an absence of these factors.

To explore this possibility, we tested UbcH5c, a promiscuous ubiquitin-conjugating enzyme (20), at an identical concentration to UBC13-UEV1A and under identical reaction conditions to determine whether its activity could be stimulated by TRAF6 (Fig. 2). We reasoned if TRAF6 oligomerization through accessory protein function is required for proper assembly into a ubiquitin ligase and substrate, reactions utilizing UbcH5c should have a defect similar to those using UBC13-UEV1A. Moreover, if TRAF6 is unable to present an appropriate nucleophile for modification, this would likely be reflected in reactions with UbcH5c due to the highly conserved enzymatic core of the ubiquitin-conjugating enzyme family (21).
In contrast to UBC13-UEV1A, UbCH5c did not detectably generate ubiquitin chains in the absence of TRAF6 (Fig. 2A, lane 1). However, its ability to synthesize ubiquitin chains was stimulated by TRAF6, similar to UBC13-UEV1A (Fig. 2A, lane 3). In examining these reactions for TRAF6 ubiquitination, we observed efficient modification of TRAF6 by UbCH5c such that the majority of TRAF6 was converted into a high molecular mass form (Fig. 2B, lane 3). Taken together, our results suggest that TRAF6 assembles into an active ubiquitin ligase complex in the absence of accessory factors in vitro. Furthermore, TRAF6 is not defective in nucleophile presentation and can serve as a substrate for modification, at least in conjunction with UbCH5c.

UBC13 Modifies TRAF6 in the Absence of UEV1A—Our experiments comparing the ubiquitin ligase activity of TRAF6 with UBC13-UEV1A or UbCH5c revealed a critical difference between these E2s. Whereas UbCH5c efficiently modifies TRAF6 and generates ubiquitin conjugates in the presence of TRAF6, it has no detectable ubiquitin-conjugating activity in its absence. UBC13-UEV1A, in contrast, generates detectable amounts of ubiquitin chains even in the absence of TRAF6 (Fig. 1B, lane 3). The presence of TRAF6 stimulates ubiquitin chain synthesis, yet very little modification of TRAF6 occurs (Fig. 1B, lane 3). As the UEV1A subunit binds a ubiquitin molecule to position Lys63 of ubiquitin near the active site cysteine of UBC13, we hypothesized that its presence may drive ubiquitin chain synthesis that in turn may suppress TRAF6 modification.

To test this hypothesis, we performed experiments in which we tested the individual subunits of the ubiquitin-conjugating enzyme in the presence of TRAF6 with either wild-type ubiquitin (Ub) or a form of ubiquitin in which all lysine residues are mutated to arginine and therefore cannot synthesize ubiquitin chains (K0 Ub). In examining ubiquitin chain synthesis in these reactions (Fig. 3A), we detected ubiquitin chain synthesis only in the presence of Ub and both subunits of the ubiquitin-conjugating enzyme (Fig. 3A, lane 3). As the pattern of ubiquitin conjugation is identical in all of these reactions, regardless of using Ub or K0 Ub, this modification is consistent with single ubiquitin attachments (i.e. “monoubiquitination”) and not ubiquitin chains. This observation is particularly striking when the ability of UbCH5c to generate high molecular weight conjuga-
Lys\(^{63}\)-linked Substrate Ubiquitination by UBC13-UEV1A

gates on TRAF6 is considered (Fig. 2B, lane 3) as it suggests UBC13 has a very specific function in substrate modification with individual ubiquitin molecules. Furthermore, these experiments suggest that a single site on TRAF6 may be the predominant site of ubiquitin attachment by UBC13, supporting recent observations by Lamonthe et al. (22) that identified a single lysine residue as important for TRAF6 function upstream of NF-κB activation.

Lys\(^{63}\) of Ubiquitin and UEV1A Stimulate Ubiquitin Chain Synthesis by UBC13 and Their Absence Promotes Substrate Modification—To test how the presence of Lys\(^{63}\) of ubiquitin affects substrate modification, we performed similar ubiquitination reactions with ubiquitin derivatives, focusing on intact (Lys\(^{63}\) only ubiquitin, with all other lysine residues mutated to arginine) or mutated Lys\(^{63}\) (K63R ubiquitin). Ubiquitin chain synthesis, as expected, requires the presence of both UEV1A and Lys\(^{63}\) of ubiquitin to occur (Fig. 3C, lanes 2 and 8), supporting the hypothesis that the positioning of Lys\(^{63}\) of ubiquitin by UEV1A near the active site cysteine of UBC13 is part of the molecular basis of ubiquitin linkage specificity and our in vitro system recapitulates this specificity.

In examining these reactions for TRAF6 modification, we observed that UBC13 alone can modify TRAF6 with a single predominant site of ubiquitin attachment with 2–4 total sites detectable for all ubiquitin derivatives tested (Fig. 3D, lanes 1, 3, 5, and 7), consistent with multiple monoubiquitination events. Similar to our results with K0 Ub (Fig. 3B), TRAF6 modification occurs in the presence of UEV1A only in the absence of Lys\(^{63}\) (Fig. 3D, lanes 4 and 6), which correlates to an observed lack of ubiquitin chain synthesis (Fig. 3C, lanes 4 and 6).

Our observation that K63R ubiquitin bound to UEV1A did not block TRAF6 modification suggests that these proteins do not simply mask sites of TRAF6 modification. Instead our data are consistent with the hypothesis that ubiquitin chain synthesis may dominate our in vitro system due to a preference for ubiquitin-ubiquitin discharge by UBC13 involving Lys\(^{63}\) of ubiquitin provided by the presence of UEV1A over TRAF6 modification.

Ubiquitin Chains Are the Major In Vitro Product Synthesized by UBC13-UEV1A in the Presence of TRAF6—To further explore the role of TRAF6 as both a ubiquitin ligase and substrate, we performed experiments examining the rate of ubiquitin chain synthesis and TRAF6 modification by UBC13-UEV1A, UbcH5c, and UBC13 alone (Fig. 4). As expected, UBC13 alone did not generate detectable ubiquitin chains in the presence of TRAF6 over the time course examined (Fig. 4A, lanes 13–18). UBC13-UEV1A (lanes 1–6) and UbcH5c (lanes 7–12), in contrast, synthesized high molecular weight ubiquitin conjugates with qualitatively similar rates of appearance.

These reactions were also analyzed for TRAF6 modification (Fig. 4B). UbcH5c modified TRAF6 such that the majority of the input substrate was converted into a high molecular weight form at the end of the time course analyzed (lanes 7–12). UBC13 alone (lanes 13–18), in contrast, converted TRAF6 considerably slower to a ubiquitinated form.

The pattern of ubiquitin incorporation onto TRAF6 by UBC13 at 20 min (lane 18) resembled the pattern generated by UbcH5c at 1 min (lane 8), suggesting that features within the largely conserved catalytic core of these E2s may contribute to their distinct ability to modify TRAF6. Whereas the molecular basis of this difference remains to be further explored, it is unlikely due to differences in the amount of active E2 in the enzyme preparations or their ability to be charged with ubiquitin as both are charged with ubiquitin by E1 with similar rates when tested at similar concentrations (Fig. 4C, compare lanes 1–6 to lanes 7–12).

As expected, UBC13-UEV1A did not efficiently modify TRAF6 when compared with either UbcH5c or UBC13 (lanes 1–6). However, longer exposures of the data (bottom panels of Fig. 4B) revealed the appearance of faint higher molecular

![FIGURE 4. UBC13-UEV1A predominantly synthesizes ubiquitin chains in the presence of TRAF6. A, ubiquitin incorporation; and B, TRAF6 modification were analyzed in reactions containing UBC13-UEV1A (lanes 1–6), UbcH5c (lanes 7–12), and UBC13 (lanes 13–18) at the time points shown. Reaction aliquots were removed at the indicated times and added to an equal volume of reducing sample buffer prior to analysis by SDS-PAGE and immunoblotting with anti TRAF6 and anti-ubiquitin antisera. The bottom panels in B are longer exposures of the top panels. The relative positions of ubiquitin (Ub), ubiquitin chains, TRAF6, and ubiquitinated TRAF6 (TRAF6-Ubn) are indicated. C, the ability of UBC13 (5 μM, lanes 1–6) and UbcH5c (5 μM, lanes 7–12) to be charged with ubiquitin (12.5 μM) was assessed in the presence of 150 nM E1 and 1 mM ATP. Reaction aliquots were removed at the indicated times and analyzed under non-reducing conditions by SDS-PAGE followed by Coomassie staining. The relative positions of free ubiquitin, unmodified UbcH5c (UbcH5c), unmodified UBC13 (UBC13), UbcH5c charged with ubiquitin (UbcH5c-Ub), UBC13 charged with ubiquitin (UBC13-Ub) and E1 are indicated.]

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weight species in the presence of UBC13-UEV1A after 5 min (compare lane 1 to lanes 4–6), consistent with ubiquitinated TRAF6.

**TRAF6 Is Monoubiquitinated by UBC13 and Modified with Ubiquitin Chains by UBC13-UEV1A**

Our data investigating ubiquitin chain synthesis and TRAF6 modification suggest a small percentage of TRAF6 is modified by UBC13-UEV1A with ubiquitin chains, we performed in vitro experiments using TRAF6 immobilized on streptavidin-Sepharose. By extensively washing Sepharose-bound material after our in vitro reactions, we could directly examine the extent of TRAF6 modification (Fig. 5).

In the absence of E2 or with UEV1A alone, we observed no modification of TRAF6 (Fig. 5A, lanes 1, 3, 5, and 7) and no detectable ubiquitin chain synthesis (Fig. 5B, lanes 1, 3, 5, and 7). As expected, TRAF6 monoubiquitination was observed with UBC13 alone in both the total reaction (soluble, Fig. 5A, lane 2) and streptavidin-bound TRAF6 after washing the Sepharose beads (bound, Fig. 5A, lane 6). By analyzing reactions under non-reducing conditions with anti-ubiquitin antisera, we could also monitor the loss of UBC13 charged with ubiquitin (UBC13-Ub thioester) from bound reactions (Fig. 5B, compare lanes 2 and 6), allowing us to assess the stringency of wash conditions as UBC13 is known to bind TRAF6 (15).

In the presence of the intact E2, we observed detectable modification of TRAF6 with ubiquitin chains (Fig. 5A, lane 4). After washing the streptavidin-Sepharose, we observed an overall background reduction, enhancing detection of TRAF6 ubiquitination by UBC13-UEV1A (Fig. 5A, lane 8). Moreover, we observed a reduction in total ubiquitin and UBC13-Ub thioester after washing (Fig. 5B, compare lanes 4 and 8) without significant reduction in the total amount of unmodified TRAF6 or ubiquitinated TRAF6 (Fig. 5A, compare lanes 4 and 8).

**Lys63-linked Substrate Ubiquitination by UBC13-UEV1A**

**Ubiquitin Discharge from UBC13 Is Stimulated by UEV1A and TRAF6**

Our data are consistent with the separation of UBC13-UEV1A activity into two distinct mechanisms, ubiquitin chain synthesis and substrate modification. TRAF6 modification with ubiquitin chains occurs at low levels in the presence of UEV1A, presumably due to more favorable positioning of Lys\(^{63}\) of ubiquitin by UEV1A relative to the UBC13 active site cysteine charged with ubiquitin than site(s) of TRAF6 modification. To explore this hypothesis, we sought to directly examine the effects of UEV1A and TRAF6 on UBC13 charged with ubiquitin (UBC13-Ub thioester). We developed an assay to examine a single turnover of ubiquitin from the active site cysteine of UBC13 (shown schematically in Fig. 6A).
Lys\textsuperscript{63}-linked Substrate Ubiquitination by UBC13-UEV1A

![Diagram](Image)

**FIGURE 6. Ubiquitin discharge from UBC13 occurs in the presence of UEV1A and TRAF6.** A, reactions to test the effect of UEV1A and TRAF6 on ubiquitin discharge from UBC13 were performed as diagrammed in the schematic shown. B, UBC13 (1 μM final concentration in discharge reaction) was charged with ubiquitin in the presence of E1, ATP, and ubiquitin, treated with the alkylating agent NEM, and then added to chase reactions to assess the stability of UBC13 charged with ubiquitin (UBC13-Ub) with respect to time. Reactions were performed in the absence of TRAF6 (−TRAF6, lanes 1–12) and the presence of TRAF6 (+TRAF6, 300 nM final concentration, lanes 13–24). The role of UEV1A on UBC13-Ub stability was also assessed (−UEV1A, lanes 1–6 and 13–18 and +UEV1A, 300 nM, lanes 7–12 and 19–24). Reaction aliquots were removed at the indicated times and added to non-reducing SDS-PAGE sample buffer prior to analysis by SDS-PAGE and immunoblotting with anti-UBC13 antisera. The incorporation of ubiquitin onto TRAF6 was analyzed in these same reactions by immunoblotting with anti-TRAF6 (anti-UBC13-Ub) antibodies. The incorporation of ubiquitin onto TRAF6 was analyzed in these same reactions by immunoblotting with anti-UBC13-Ub antibodies.

**A**

- E1
- Ub
- ATP
- NEM
- +/− UEV1A
- +/− TRAF6
- UBC13
- Charge
- Stop
- Chase
- Analyze UBC13-Ub, UBC13

**B**

- Time (min):
- 0 1 2 5 10 20
- 1 2 5 10
- 0 1 2 5 10 20
- -UEV1A +UEV1A
- -TRAF6 +TRAF6
- UBC13-Ub

**C**

- E1
- FLAG-Ub
- ATP
- +/− NEM
- +/− UEV1A
- +/− TRAF6
- UBC13
- Charge
- Stop
- Chase
- Analyze UBC13-Ub (anti-FLAG)

**D**

- Time (min):
- 0 1 2 5 10 20
- 0 1 2 5 10 20
- -NEM +NEM
- -UEV1A +UEV1A
- -TRAF6 +TRAF6
- UBC13-Ub

from UBC13 was monitored under non-reducing conditions from reaction aliquots removed at the indicated times by blotting these reactions with anti-FLAG conjugated to horseradish peroxidase. As ubiquitin discharge from UBC13 appeared similar in these reactions, the presence of NEM in the discharge reaction has no significant downstream effect.

**UEV1A More Efficiently Stimulates Ubiquitin Discharge from UBC13-UB Thioester Than TRAF6**—One possible explanation for observable differences in the rate of UBC13-UB thioester discharge upon addition of either TRAF6 or UEV1A could be that UBC13-UB thioester interacts more efficiently with UEV1A than TRAF6. This combined with the effects of UEV1A positioning Lys\textsuperscript{63} of ubiquitin promotes covalent ubiquitin transfer by UBC13-UB thioester onto Lys\textsuperscript{63} of ubiquitin better than onto the sites of TRAF6 modification. To explore these possibilities, we performed titration experiments in which the molar ratio of UEV1A or TRAF6 was varied with respect to a fixed concentration of UBC13 (500 nM final concentration in the discharge reaction). We examined UBC13-UB thioester discharge with respect to time after NEM treatment and upon addition of these variable concentrations of UEV1A or TRAF6 (shown schematically in Fig. 7A).

As expected, increasing the concentration of UEV1A resulted in an increase in the rate of UBC13-UB thioester discharge to an observable maximum as the UEV1A binding site on UBC13-UB thioester becomes saturated (Fig. 7B). Under limiting UEV1A conditions (1:2.5 with respect to UBC13, lanes 1–6), UEV1A must interact with multiple UBC13-UB thioester molecules to promote ubiquitin discharge as the absence of UEV1A did not promote efficient discharge from UBC13-UB thioester (Fig. 6B). We observed no further increases in the rate of UBC13-UB thioester loss and concomitant appearance of UBC13 above a 2.5-fold molar excess of UEV1A relative to UBC13. As these reactions contain excess free ubiquitin to saturate the ubiquitin binding site on UEV1A (see “Experimental Procedures” for experimental details), the time required for UBC13-UB thioester discharge likely reflects the combination of the binding of UBC13-UB thioester to UEV1A, the nucleophilic attack by Lys\textsuperscript{63} of the UEV1A-bound ubiquitin of the ubiquitin on the active site of UBC13-UB thioester, and covalent ubiquitin transfer.

Similar to experiments testing UEV1A, increasing the concentration of TRAF6 in the discharge reactions increased the rate of UBC13-UB thioester discharge (Fig. 7B). Whereas a 2.5-fold molar excess of UEV1A to UBC13 resulted in a maximal rate of UBC13-UB thioester discharge (Fig. 7B, lanes 1–6), a 5-fold molar excess of TRAF6 relative to UBC13 resulted in maximal rates of UBC13-UB thioester discharge (Fig. 7B, lanes 43–48). Technical limitations prevented us from testing higher concentrations of TRAF6 under these experimental conditions. However, at lower concentrations of UBC13, we did not observe differences in the rate of UBC13-UB thioester discharge above a 5-fold molar excess of TRAF6 (data not shown),
suggesting that the TRAF6 binding site on UBC13 is saturated at a 5-fold molar excess of TRAF6 relative to UBC13 in these single turnover experiments.

**DISCUSSION**

Our data are consistent with the ubiquitin-conjugating enzyme UBC13-UEV1A fulfilling two distinct roles; ubiquitin chain synthesis and substrate modification, dependent upon the presentation of Lys63 of ubiquitin by UEV1A (see model in Fig. 8A). UEV1A drives ubiquitin chain synthesis by properly presenting Lys63 of a bound ubiquitin molecule near the active site cysteine of UBC13. Substrate modification, in contrast, occurs less efficiently in the presence of UEV1A and ubiquitin containing lysine 63, suggesting a kinetic separation between chain synthesis and substrate modification. Ubiquitin chain synthesis largely predominates our reactions when UEV1A binds a ubiquitin molecule. We propose this mechanism ensures that regulation afforded by distinct ubiquitin chain topology proceeds with the appropriate temporal control.

We observed efficient monoubiquitination of TRAF6 in vitro by UBC13 in the absence of UEV1A relative to the modification of TRAF6 with ubiquitin chains in reactions employing UBC13-UEV1A (see Fig. 4B, for example). This difference does not reflect impairment of enzyme function in the presence of UEV1A as we observed the conversion of ubiquitin into higher molecular weight forms similar to reactions utilizing UbcH5c (Fig. 4A). Rather, inefficient ubiquitination of TRAF6 likely reflects the preference of UBC13 to discharge ubiquitin onto Lys63 of ubiquitin bound by UEV1A. We provide evidence supporting this hypothesis in Fig. 6, as UEV1A stimulates ubiquitin discharge from UBC13-Ub thioester to a greater extent than TRAF6. Free ubiquitin in our in vitro reactions likely drives unanchored ubiquitin chain synthesis by UBC13-UEV1A as UEV1A binds ubiquitin with a measured dissociation constant of ~213 μM (10).

Our observations that UBC13 alone or UBC13-UEV1A in the presence of ubiquitin lacking Lys63 can monoubiquitinate TRAF6 complement a recent study by Lamothe et al. (22). In this work, a single site on TRAF6 (Lys124) was reported to be sufficient for ubiquitin-dependent IKK activation (22). Mutation of this site correlated to both a reduction of TRAF6 ubiquitination and TAK1 activation. Our kinetic analysis (Fig. 4B) demonstrates an initial predominant single site modification of TRAF6 by UBC13 with other slower appearing secondary attachment sites. Although we do not precisely map the sites of modification here, it is intriguing to speculate that our in vitro system employs site-specific modification observed in these cell-based experiments. Future studies based on our initial characterization of this in vitro system will likely allow us to address the minimal requirements and molecular mechanism underlying activation of the kinase TAK1 by Lys63-linked ubiquitin chain modification of TRAF6.

The monoubiquitination of TRAF6 has not been observed in cells and UBC13 is thought to exist exclusively as a heterodimer with its UEV subunits such as MMS2 and UEV1A (4, 7–12). Thermodynamic and kinetic analyses of the UBC13-UEV interaction revealed a strong interaction between the two subunits (dissociation constant of 49 nM) (10). A large hydrophobic interface, estimated at 1500 Å, may be largely mediated through a single phenylalanine residue on the UEV subunit (amino acid 13 in MMS2 and 38 in UEV1A) that inserts into a hydrophobic pocket on UBC13 (7). The UEV subunit in the context of the heterodimeric UBC13-UEV complex binds ubiquitin more tightly than as a free subunit in vitro (28 versus 98 μM dissociation constants, respectively; measured for MMS2 (10)). Based on these observations, we propose that substrate ubiquitination through the activity of UBC13-UEV requires that the UEV subunit is not initially associated with ubiquitin exposing an unmodified Lys63 primary amine side chain. In light of this hypothesis, our data are consistent with two models (schematically shown in Fig. 8A): ubiquitin chains are pre-assembled and added as a unit by UBC13 onto the ubiquitin ligase-bound substrate or ubiquitin chains are sequentially added with an initial substrate-ubiquitin attachment followed by UEV1A-dependent chain extension, similar to models recently proposed by Hochstrasser (24).

For a pre-assembly model, Lys63-linked ubiquitin chain synthesis can occur both in the presence or absence of the ubiquitin ligase. Our data (Fig. 1) suggest that the addition of TRAF6

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**FIGURE 7.** UEV1A and TRAF6 stimulate ubiquitin discharge from UBC13 to different extents. A, reactions to assess the effect of varying ratios of either UEV1A or TRAF6 relative to a fixed concentration of UBC13 (500 nM) were performed as diagrammed in the schematic shown. B, UEV1A (lanes 1–6, 13–18, 25–30, and 37–42) or TRAF6 (lanes 7–12, 19–24, 31–36, and 43–48) were added at the indicated molar ratios with respect to UBC13 after UBC13 had been pre-charged with ubiquitin and treated with NEM/EDTA. Reaction aliquots were removed at the indicated times and analyzed by SDS-PAGE and immunoblotting with anti-UBC13 antisera.
to reactions enhances the basal level of UBC13-UEV1A to synthesize Lys63-linked ubiquitin chains. Molecular modeling (Fig. 8, B and C) suggests that the distance between the active site cysteine of UBC13 (Cys87) and the primary amine of the Lys63 side chain of the non-covalently bound ubiquitin molecule on UEV1A is 9.8 Å. The binding of UBC13-UEV1A to TRAF6 may decreases this distance, potentially through an allosteric mechanism as proposed by Ozkan et al. (25) for UbcH5c, resulting in enhanced Lys63-linked ubiquitin chain synthesis. An alternative hypothesis is that TRAF6 may potentially promote Lys63 de-protonation of the ubiquitin molecule bound to UEV1A. Regardless of the exact molecular details, pre-assembled Lys63-linked ubiquitin chains would be directly added as a unit by UBC13 onto substrates like TRAF6 and immediately be competent to fulfill its signaling functions such as the activation of TAK1. UEV1A may also have a secondary role in promoting substrate modification by positioning the pre-assembled ubiquitin chain to block subsequent ubiquitin chain extension and as a result facilitate substrate modification.

A sequential addition model requires the initial absence of Lys63 of ubiquitin presented by UEV1A to UBC13-UEV1A thioester, similar to the pre-assembly model. After the rate-limiting addition of the initial ubiquitin molecule onto the substrate, ubiquitin chain elongation may proceed through UEV1A-dependent recognition of the priming ubiquitin on the substrate and positioning of Lys63 near the active site cysteine of UBC13-UEV1A thioester. This would promote Lys63-linked ubiquitin chain extension through the function of UEV1A and would proceed through a more rapid mechanism than the initial ubiquitin addition.

These models are obviously difficult to distinguish experimentally and therefore establish conclusively. Nevertheless, our present work establishes a paradigm for the mechanism underlying substrate modification with Lys63-linked ubiquitin chains through UBC13-UEV1A with a ubiquitin ligase. The presence of Lys63 of ubiquitin bound to the UEV subunit serves to drive ubiquitin chain synthesis and impair substrate modification. Thus, the kinetic partitioning of substrate modification and ubiquitin chain synthesis is largely defined by the role of UEV1A and may ensure that the signals encoded by Lys63-linked ubiquitin chains are efficiently transmitted to promote downstream processes like kinase activation.

Mechanisms separating substrate modification and ubiquitin chain synthesis do not appear to be restricted to only UBC13-UEV1A-dependent pathways. Recent biochemical studies on the E2 Cdc34 and its ubiquitin ligase SCF led to the proposal that ubiquitination of the budding yeast SCF substrate Sic1 occurs in distinct steps, the initial attachment of ubiquitin onto Sic1 and subsequent lysine 48-linked ubiquitin chain extension (23). A unique region near the active site cysteine of Cdc34, termed the acidic loop, appears to be largely responsible for stimulating ubiquitin chain synthesis yet has no discernible role on the initial substrate-ubiquitin attachment. Sic1 contains multiple lysine residues of which an individual lysine is sufficient to promote in vivo turnover and in vitro ubiquitination and degradation in a purified system (26). Thus, it was proposed that the kinetic separation between initial rate-limiting ubiquitin attachment and subsequent rapid Lys48-linked ubiquitin chain extension ensures that only the minimum targeting signal, a single ubiquitin chain, is attached onto a given protein substrate (23).
A recent screen for the E2 that function with the yeast anaphase promoting complex ubiquitin ligase identified important roles for both Ubc4 and Ubc1 (27). Characterization of these two enzymes revealed that Ubc4 promotes the modification of lysine residues on anaphase promoting complex substrates with individual ubiquitin molecules and Ubc1 (the yeast homologue of human E2–25K) synthesizes Lys48-linked ubiquitin chains. The authors proposed that sequential E2 function, Ubc4 adding the initial ubiquitin to an anaphase promoting complex substrate followed by Ubc1 extension via Lys48-based ubiquitin linkages, underlies how these substrates are targeted for degradation by the proteasome.

The Kaposi sarcoma-associated herpesvirus ubiquitin ligase K3 may also utilize dual ubiquitin-conjugating enzyme functions to ubiquitinate cell surface major histocompatibility complex class I molecules and target them to the lysosome (28). Cell-based small interfering RNA studies identified both UbcH5 and UBC13 as required for this down-regulation. It was hypothesized by the authors that UbcH5 initially modifies major histocompatibility complex class I molecules recognized by Lys3 with monoubiquitin followed by UBC13-dependent Lys63-linked ubiquitin chain extension.

In summary, the data presented here reveal the mechanistic basis of how UBC13-UEV1A functions with its ubiquitin ligases to modify substrates and synthesize ubiquitin chains. Whereas both sites on TRAF6 and Lys63 of ubiquitin bound to UEV1A facilitate ubiquitin discharge from UBC13, they have differential effects. Lys63-linked ubiquitin chain synthesis is an intrinsic feature of UBC13-UEV1A and the presence of the ubiquitin ligase stimulates this activity. In contrast, the modification of TRAF6 appears to be kinetically slower and therefore may represent the rate-limiting step in protein ubiquitination.

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