Tripartite Motif Ligases Catalyze Polyubiquitin Chain Formation through a Cooperative Allosteric Mechanism*

Frederick C. Streich, Jr. 1, Virginia P. Ronchi 2, J. Patrick Connick 2, and Arthur L. Haas 1,2

From the 1Department of Biochemistry and Molecular Biology and the 2Stanley S. Scott Cancer Center, Louisiana State University Health Sciences Center, New Orleans, Louisiana 70112

Ligation of polyubiquitin chains to proteins is a fundamental post-translational modification, often resulting in targeted degradation of conjugated proteins. Attachment of polyubiquitin chains requires the activities of an E1 activating enzyme, an E2 carrier protein, and an E3 ligase. The mechanism by which polyubiquitin chains are formed remains largely speculative, especially for RING-based ligases. The tripartite motif (TRIM) superfamily of ligases functions in many cellular processes including innate immunity, cellular localization, development and differentiation, signaling, and cancer progression. The present results show that TRIM ligases catalyze polyubiquitin chain formation in the absence of substrate, the rates of which can be used as a functional readout of enzyme function. Initial rate studies under biochemically defined conditions show that TRIM32 and TRIM25 are specific for the Ubc5 family of E2-conjugating proteins and, along with TRIM5α, exhibit cooperative kinetics with respect to Ubc5 concentration, with submicromolar [S]0.5 and Hill coefficients of 3–5, suggesting they possess multiple binding sites for their cognate E2-ubiquitin thioester. Mutation studies reveal a second, non-canonical binding site encompassing the C-terminal Ubc5α-helix. Polyubiquitin chain formation requires TRIM subunit oligomerization through the conserved coiled-coil domain, but can be partially replaced by fusing the catalytic domain to GST to promote dimerization. Other results suggest that TRIM32 assembles polyubiquitin chains as a Ubc5-linked thioester intermediate. These results represent the first detailed mechanistic study of TRIM ligase activity and provide a functional context for oligomerization observed in the superfamily.

Post-translational conjugation of ubiquitin and ubiquitin-like proteins to polypeptide targets provides a diverse set of functional outcomes that regulate most aspects of cell homeostasis. The best understood of these functions is assembly of polyubiquitin chains on protein targets, resulting in degradation of the conjugated substrate by the 26S proteasome and disassembly of the polyubiquitin degradation signal (1). Through such targeted degradation, the ubiquitin system is a critical mechanism by which cells regulate steady-state protein concentrations and cellular homeostasis (2–4). Ligation of ubiquitin to target proteins functions through a conserved mechanism involving the sequential activities of three enzymes that catalyze new isopeptide bond formation between the C terminus of ubiquitin and primary amines present on the target (2, 5). The activating enzyme (E1) 3 couples ATP hydrolysis to activation of the C terminus of ubiquitin to form a ternary complex comprising both E1-ubiquitin thioester and tightly bound ubiquitin adenylyl moieties (6). The E1 ternary complex then binds any of a superfamily of cognate E2 proteins to catalyze transthioleolysis of the ubiquitin thioester between the conserved active site cysteines of E1 and E2 (7, 8). The second half-reaction of target protein ligation is then catalyzed by one of several hundred E3 ligases that couples new isopeptide bond formation to the aminolytic cleavage of the high energy E2-ubiquitin thioester (2).

Less understood is the mechanism by which polyubiquitin chains are synthesized (9), particularly among RING ligases that constitute a diverse group of conjugating enzymes sharing a common catalytic domain that coordinates two Zn2+ ions to stabilize specialized RING finger motifs (4). The RING domains constitute the functional cassette that is responsible for binding the cognate E2-ubiquitin thioester that functions as a co-substrate for the ligase and for catalysis of isopeptide bond formation (4). In the absence of substrate, RING ligases catalyze slow formation of high molecular weight free and autoconjugated.

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1 Present address: Research Program in Structural Biology, Sloan-Kettering Institute, Memorial Sloan Cancer Center, 1275 York Ave., New York, NY 10065.
2 To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center, New Orleans, LA 70112. Tel.: 504-568-3004; Fax: 504-568-2093; E-mail: ahaas@lsuhsc.edu.

3 The abbreviations used are: E1, ubiquitin activating enzyme; E3, ubiquitin-protein isopeptide ligase; E2/Ubc, ubiquitin carrier protein or ubiquitin conjugating enzyme; NHL, protein domain from representative proteins NCL-1, HT2A, and LIN-41; RING, Really Interesting New Gene protein domain; TRIM, tripartite motif; Uba1, human ubiquitin activating enzyme.
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polyubiquitin chains (10, 11). Observation of free polyubiquitin chain formation challenges the conventional view that chains are assembled on the target substrate sequentially by distal addition of ubiquitin moieties to a growing target-anchored ubiquitin chain (9). However, a mechanism proposed for substrate conjugation by the gp78 ligase in which polyubiquitin chains are first assembled on the cognate Ube2g2/Ubc7 ubiquitin carrier protein then transferred to the target protein en bloc anticipates free chain formation as the result of the side reaction of E2-chain thioester hydrolysis (12).

The tripartite motif (TRIM) superfamily constitutes the largest group of RING ligases with over 100 paralogs (13, 14). Members of the TRIM superfamily share a common tripartite RING-B box-coiled coil domain architecture and function in various aspects of cell regulation, development, innate immunity, and the etiology of certain cancers (13, 15). The B box and coiled coil domains are required for oligomerization of TRIM family members, although the role of oligomerization in the function of these enzymes is unclear (13). Additional domain(s) C-terminal to the coiled coil domain serve as targeting sites for recruiting protein substrates for ubiquitin conjugation (13). As with other RING ligases, the mechanism by which the TRIM paralogs catalyze formation of the polyubiquitin degradation signal is thought to proceed through sequential distal addition of ubiquitin to the growing chain appended to the target protein (2, 13), although this mechanism has not been empirically tested.

The current study investigates the mechanism of polyubiquitin chain formation catalyzed by TRIM32, the function of which is linked to tumorigenesis in squamous cell carcinoma and other cancers (16–18), the etiology of psoriasis (19), Alzheimer syndrome (20), and neuronal development/survival (21, 22). Defects in girdle muscular dystrophy (23) and Bardet-Biedl syndrome (21, 22). Defects in Alzheimer syndrome (20), and neuronal development/survival (21, 22). Defects in girdle muscular dystrophy (23) and Bardet-Biedl syndrome (24). We have exploited the innate ability of TRIM32 to catalyze polyubiquitin chain formation in the absence of substrate as a functional readout of ligase activity (10). Selected experiments have been extended to two other members of the TRIM ligase superfamily to explore the generality of our observations. In this first mechanistic examination of TRIM ligase function, kinetic studies suggest that members of the TRIM superfamily function through a conserved allosteric cooperative mechanism requiring oligomerization. Additional rate data are consistent with an elongation mechanism in which polyubiquitin chains are first assembled as an E2-linked thioester prior to being appended en bloc to the target protein.

MATERIALS AND METHODS

Bovine ubiquitin, creatine phosphokinase, and apyrase were purchased from Sigma. Ubiquitin was further purified to apparent homogeneity (25) then radiolabeled by the chloramine-T procedure using carrier-free Na125I (PerkinElmer Life Sciences) to yield a specific radioactivity of 8000 cpm/pmol (26). Thrombin was purchased from GE Healthcare. Human erythrocyte Uba1 (UBE1) was purified to apparent homogeneity from outdated human blood (27). Active Uba1 was quantitated by the stoichiometric formation of 125I-ubiquitin thioester (27, 28). Recombinant human E2 proteins were those used previously (29), the activities of which were quantitated by the stoichiometric formation of E2-125I-ubiquitin thioester and compared with the expected activities based on protein content determined by their calculated 280 nm extinction coefficients (10, 28). Point mutants of Ubc5A were created using the QuikChange kit (Stratagene) (29, 30). Recombinant human IsoT (USP5) was expressed in Escherichia coli BL21(DE3) cultures harboring pRSIsoS (generous gift of Dr. Keith Wilkinson, Emory University School of Medicine) and purified to apparent homogeneity as described previously (31). The concentration of IsoT in the incubations was calculated from total protein determined spectrophotometrically using a theoretical ε280 nm extinction coefficient of 1.09 mg/ml⁻¹.

Expression and Purification of Recombinant TRIM Proteins—Human TRIM32 cDNA (TRIM32; IMAGE clone BC003154) was used as a template for PCR-based subcloning of the full-length and truncated forms of the coding sequence into pPRO-EX-HTb and pGEX-4T1 vectors using the EcoRI/XhoI restriction sites. Bovine ubiquitin, creatine phosphokinase, and apyrase were purchased from GE Healthcare. Human erythrocyte Uba1 (UBE1) was purified to apparent homogeneity (25) then radioiodinated by the chloramine-T mechanism requiring oligomerization. Additional rate data are consistent with an elongation mechanism in which polyubiquitin chains are first assembled as an E2-linked thioester prior to being appended en bloc to the target protein.
was cloned from pCDN3.1-Trim5α (generous gift of Dr. Ronald Luftig, Louisiana State University School of Medicine) into pGEX4T1 using the EcoRI/Xhol restriction sites to yield pGEX4T1-Trim5α. Recombinant Trim5α was expressed in the Rosetta strain of E. coli BL21 cells (Millipore) and purified identically to Trim25.

In Vitro Polyubiquitin Chain Formation Assays—Formation of 125I-ubiquitin chains was measured at 37 °C in 25-μl reactions containing 50 mM Tris-HCl (pH 7.5), 2 mM ATP, 10 mM MgCl₂, 10 mM creatine phosphate, 0.5 IU of creatine phosphokinase, 1 mM DTT, 0.1 mg/ml of BSA, 5 mM 125I-ubiquitin, and the indicated concentrations of Uba1, E2, and TRIM protein. After 10 min, reactions were quenched by addition of 25 μl of SDS sample buffer, then incubated at 100 °C for 3 min and resolved by 12% (w/v) SDS-PAGE (10, 28). The resulting 125I-polyubiquitin chains were visualized by autoradiography of the dried gels then, unless otherwise indicated, associated radioactivity in the stacker corresponding to free polyubiquitin chains was determined by excising the regions of conjugates and quantitating associated radioactivity by γ-counting (10, 32). Kinetic assays measured the initial rates of chain formation, for which less than 5% of total 125I-ubiquitin was conjugated, and under E3-limiting conditions, confirmed by the independence of rate on [Uba1]₀ (10). Sigmoidal kinetics for Trim32-catalyzed chain formation were verified by the linearity of the resulting Hill plot; however, values for [S]₀.5 and the Hill coefficient n were calculated by nonlinear regression analysis using Grafit 5.0 (Erithacus Software Ltd.).

IsoT-catalyzed Polyubiquitin Disassembly—In vitro chains were formed as described above and reactions were quenched with 1 IU of apyrase for 5 min at 37 °C quantitatively to deplete ATP before adding DTT to a final concentration of 10 mM and incubating an additional 5 min at 37 °C to cleave polyubiquitin chains from their E2 thioester linkage. To this reaction apparently homogeneous recombinant IsoT was added to a final concentration of 100 μM (zero time) after which aliquots were removed at the indicated times and quenched with an equal volume of SDS sample buffer. After 60 min, a second aliquot of IsoT was added and incubation was continued an additional 30 min. Samples were resolved by SDS-PAGE and autoradiographed.

Assembly of Polyubiquitin Chains on Ubc5AC85S-125I-Ubiquitin Oxyster—The Ubc5AC85S-125I-ubiquitin oxyster was synthesized and purified by Superdex 75 FPLC on a 1 × 30-cm column as described previously for Ubc2bC88S-125I-ubiquitin oxyster (33). Eighty nm Ubc5AC85S-125I-ubiquitin oxyster was incubated at 37 °C in a 30-μl volume containing 50 mM Tris-HCl (pH 7.5), 2 mM ATP, 10 mM MgCl₂, 10 mM creatine phosphate, 0.5 IU of creatine phosphokinase, 1 mM DTT, 0.1 mg/ml of BSA, 80 nm Uba1, 100 nm Ubc5A, 10 μM Trim32 protein, and 5 μM ubiquitin (as indicated) for 10 min. Reactions were quenched with SDS sample buffer and resolved by 12% SDS-PAGE. The gel was dried and bands were visualized by autoradiography, excised from the gel, and associated radioactivity was quantitated by γ-counting (10, 32).

Glutaraldehyde Cross-linking—Oligomerization of Trim32 and GST-TRIM were demonstrated by glutaraldehyde cross-linking essentially as described by Peng et al. (34). Reactions of 20 μl final volume containing 50 mM Tris-HCl (pH 7.5), the indicated concentrations of glutaraldehyde, and either 2.9 μM TRIM32ΔNHL or 2.3 μM GST-RING were incubated 5 min at 22 °C before quenching by addition of 20 μl of SDS sample buffer. Reactions were then incubated an additional 30 min at 37 °C before resolving at 12% (w/v) SDS-PAGE. Proteins were electrotherophoretically transferred to PVDF membrane (Millipore) and detected with rabbit anti-human Trim32 antibody directed against the RING domain (Abcam, ab50555). Bound antibody was visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and Pierce ECL Western blot substrate (Thermo Scientific) according to the manufacturer’s instructions.

RESULTS

Conservation of E2 Specificity within the TRIM Ligase Family—An important step in the functional characterization of ubiquitin ligases requires identification of their cognate E2 for supporting the conjugation reaction because ligases typically function efficiently with members of only a single E2 family (10). However, such identification is not trivial because the E2 proteins share similar amino acid sequences and folds within their highly conserved ~150 residue core catalytic domains (5, 35). These inherent structural similarities result in the potential for spurious binding interactions between E2 and E3 proteins at high concentrations of the interactors or long incubation times that frequently lead to ambiguities in the literature regarding correct E2-E3 cognate pairs, as has been discussed previously (5, 10). Trim32 is reported to function with isoforms of the Ubc5 family of E2 proteins and the unrelated UbcH6 family (36, 37). To quantitatively re-examine the question of the cognate E2 for Trim32, the biochemically defined functional E2 screen of Fig. 1 was performed under E3 limiting initial velocity conditions and equivalent 50 nm concentrations of active E2 protein, determined in activity assays by their stoichiometric formation of 125I-ubiquitin thioester (10, 28). The former conditions guarantee that the resulting autoradiographic intensity for conjugates formed in the reaction reflect a relative rate, whereas the latter functional assays obviate variations in E2 concentration originating from differences in specific activities of the E2 proteins when defined by total protein (10).

No conjugation was observed in the presence of Uba1 and Trim32 alone (Fig. 1A, lane 2). Significant conjugation is observed only upon addition of the Ubc5 isoforms (Fig. 1A, lanes 4–6), indicating that these paralogs are the cognate E2 species for Trim32. Conjugation supported by the Ubc5 isoforms is sufficiently robust that a significant fraction of the adducts appear at the top of the resolving gel or fail to migrate into the 5% (w/v) stacker (indicated). The minor 125I-ubiquitin adduct bands below 50 kDa observed with some E2 species result from E3-independent E2 autoubiquitination (10). The inability of an equivalent concentration of active UbcH6 to similarly support Trim32 conjugation (lane 7) suggests that the E3-independent autoubiquitination noted earlier for this paralog (29) may have been mistaken for Trim32-catalyzed conjugation or that the concentration of UbcH6, determined from the protein concentration (37), was sufficiently high to force an otherwise low affinity binding interaction not detected under the current biochemically defined conditions. The E3-depen-
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FIGURE 1. E2 screen for TRIM32-catalyzed 125I-ubiquitin conjugation. A, autoradiogram of SDS-PAGE-resolved assays of TRIM32-catalyzed 125I-ubiquitin chain formation. Assays were conducted as described under "Materials and Methods" in the presence of 44 nM Uba1, 50 nM of the indicated E2, and 800 nM TRIM32. B, autoradiogram of SDS-PAGE resolved assays conducted identically to panel A but in the presence of 1.6 μM TRIM23 or 27 μM GST-RING protein and 460 nM Ubc5A as indicated. Incubations additionally contained 5 μM wild type (left subpanel) or reductively methylated (rmUb, right subpanel) 125I-ubiquitin. The left and right panels were exposed to normalize to account for the difference in specific radioactivity of the polypeptides. For panels A and B, migration positions for relative molecular weight standards are shown to the left. The position of the 5% stacker gel (Stacker), monoubiquitinated TRIM32 (Mw,calc = 44 kDa), monoubiquitinated Ubc5A (Mw,calc = 25 kDa), and free 125I-ubiquitin are shown to the right. Deviation of the apparent relative molecular weight of the monoubiquitinated species from their calculated molecular masses (Mw,calc) reflects non-ideality of ubiquitin due to its partial unfolding in SDS.

The ability of TRIM32 to form both free and anchored chains is demonstrated by the disappearance of a portion of the high molecular weight 125I-ubiquitin signal upon treatment with the ubiquitin-specific protease IsoT, which disassembles only free polyubiquitin chains (39) (Fig. 2, A and C). At the long exposure use in panel A of Fig. 2 to emphasize the labile signal in the stacker, one also observes loss of the lower molecular mass signal immediately above 25 kDa upon incubation with IsoT (lane 4 versus 5–7). That a fraction of the chains are refractory to disassembly by IsoT, even after further incubation with additional enzyme (Fig. 1C, open circle, and Fig. 2A, lane 7), demonstrates that ~50% of the signal is due to anchored chains under these conditions, presumably representing autoubiquitination of TRIM32.

We also tested the specificity of TRIM25, which has been reported to function with a variety of E2 paralogs including UbcH8 (40, 41), Ubc5A (42, 43), Ubc5C (44), UbcH6 (41), and Ubc13 (43), all of which are members of the Ubc4/5-like clade (5). An E2 screen identical to that of Fig. 1A demonstrates that TRIM25-catalyzed polyubiquitin chain formation is supported exclusively by members of the Ubc5 family (not shown). Finally, a third E2 screen with recombinant TRIM5α also confirmed an earlier report that the ligase functions only with the three Ubc5 isozymes (45), not shown. Thus, of the three representative TRIM paralogs tested, all are supported exclusively by the Ubc5 family of ubiquitin carrier proteins, suggesting Ubc5 ortholog specificity may be conserved among the TRIM family members.

Polyubiquitin Chain Formation Requires Oligomerization of the RING Domain—The RING domain is conserved among nearly all of the TRIM family members (13, 46) as well as several hundred other ligases possessing distinct domain architectures (4). A panel of truncated TRIM23 proteins was generated to investigate the minimum portion of TRIM32 necessary to form polyubiquitin chains, schematically summarized in Fig. 3C. Conjugation assays similar to those of Fig. 1A were conducted under E3-limiting initial velocity conditions so that the autoradiographic intensity of the resulting autoradiogram would be proportional to rate (10). Additionally, assays were performed at different E3 concentrations to ensure the conjugation observed was E3 limiting, as shown by the proportionality of rate to the TRIM construct concentration. Fig. 3A demonstrates that truncation of the NHL domains has no effect on polyubiquitin chain formation because equimolar TRIM32 or TRIM32ΔNHL qualitatively exhibits nearly identical rates of 125I-ubiquitin chain formation (Fig. 3A, lanes 7, 8, 11, and 12) compared with controls with Uba1 and Ubc5A alone (lane 2) or Uba1 and TRIM32 alone (lanes 3–6). However, additional truncation of the B box and coiled coil domains to yield the RING domain alone abrogates chain formation (lanes 9 and 13). The ability to form chains is partially rescued by fusion of the free RING domain to GST (lanes 10 and 14), presumably due to the propensity of GST to dimerize (47, 48) and partially substitute for B box- and coiled coil domain-mediated oligomerization (46). Apparent loss of chain formation following in situ processing of GST from the RING (Fig. 3B, lane 5 versus 6) is not due to artificial processing the polyubiquitin chains by
thrombin because the protease has no effect on chains when added after the chain formation reaction has been quenched (Fig. 3B, lane 7). Nor is loss of chain formation due to direct inhibition by thrombin, because the GST–RING ruined thrombin fusion protein harboring a mutated thrombin cleavage site is not similarly inhibited by thrombin treatment (lane 9).

Although the processed RING domain alone is incapable of catalyzing polyubiquitin chain formation, it is able to undergo automonoubiquitination to yield a unique band of about 18 kDa (Fig. 3B, lane 6), formation of which can serve as a functional readout of RING domain activity.

To confirm that fusion of GST to the RING domain rescues the functional phenotype of chain formation observed for full-length TRIM32, the activity of the GST–RING fusion was tested in the presence of 125I-ubiquitin. Substitution of 125I-ubiquitin for wild type 125I-ubiquitin in the GST–RING-catalyzed chain formation assay results in significant loss in conjugation, indicat-

Nonlinear regression fit of the data in Fig. 4B to the Hill equation allows calculation of $V_{\text{max}}$ (0.54 ± 0.01 pmol/min), the corresponding $k_{\text{cat}}$ as $V_{\text{max}}/K_{m}$ (4.5 ± 0.8 × 10⁻³ s⁻¹) with $K_{m}$ expressed as total protein, the concentration of substrate yielding half-maximal velocity [S]₀₅₀ (410 ± 10 nm), and the Hill coefficient $n$ (5.1 ± 0.3) (Table 1). Parallel experiments similar to that of Fig. 4 revealed that TRIM25 and
TRIM5α also exhibit cooperativity with respect to [Ubc5A], and substrate inhibition at higher concentrations of the E2 (not shown). The resulting kinetic constants derived from nonlinear regression analysis of the respective data also corresponded well to that obtained with TRIM32 (Table 1). This suggests that cooperativity and the mechanism of polyubiquitin chain formation are conserved catalytic features of the TRIM superfamily.

Allosteric cooperativity was observed with TRIM32ΔNHL (not shown), consistent with the ability of the TRIM enzymes to oligomerize through their conserved B box-coiled coil domains in the absence of the NHL targeting domains and yielded kinetics similar to wild type TRIM32 when normalized to enzyme concentration (Table 1). To confirm that subunit association through the B box-coiled coil region is required for the cooperative allosteric response, we also examined the kinetics of a GST-RING construct derived from the catalytic domain of TRIM32 (not shown) and the corresponding free RING domain tethering the RING domains is substituted by fusion to GST, which is known to dimerize with micromolar affinity (34, 50). In contrast, the free RING domain generated by in situ thrombin processing catalyzes only automonoubiquitination, suggesting oligomerization is required for chain formation (Figs. 2, B, lane 6, and 4C). When the initial rate of monoubiquitination is exploited as a functional readout of free RING domain catalytic activity, this module exhibits hyperbolic kinetics with respect to [Ubc5A], \( K_m = 630 \pm 286 \, \text{nm} \) and \( k_{cat} = 8.2 \pm 1.9 \times 10^{-6} \, \text{s}^{-1} \), confirmed by the linearity of the corresponding double-reciprocal plot (Fig. 4D, inset).

Glutaraldehyde cross-linking experiments were performed on TRIM32ΔNHL and GST-RING proteins to determine the number of subunits present in the active E3. Consistent with previous observations for TRIM5α, TRIM28/KAP1, and TRIM33/TIF1α/γ (51, 52), glutaraldehyde cross-linking experiments demonstrate a fraction of the TRIM32ΔNHL and GST-RING proteins can be covalently cross-linked (Fig. 5). Previously, Li et al. (51) observed cross-linking that they described as consistent with a trimer, whereas Peng et al. (52) observed even higher oligomers. In the present studies, cross-linking of TRIM32ΔNHL or GST-RING yield bands with relative molecular weights consistent with dimers, tetramers, and higher order oligomers (Fig. 5), supporting levels of oligomerization of TRIM32 predicted by the observed Hill coefficient (Table 1).

**FIGURE 3.** Polyubiquitin chain formation requires multimerization. A, incubations similar to those of Fig. 1 were conducted with TRIM32 (lanes 7 and 11), TRIM32ΔNHL (lanes 8 and 12), RING domain alone formed by processing with 90 IU/ml of thrombin for 30 min (lanes 9 and 13), or a GST-RING fusion (lanes 10 and 14). Reactions contained 48 nM Uba1 and either 450 (left subpanel) or 100 nM (right subpanel) of the indicated E3 protein in the absence (lanes 3–6) or presence (lanes 7–14) of 280 nM Ubc5A. B, thrombin-dependent processing of GST from the RING moiety is responsible for abrogation of chain formation. The GST-RING or GST-RING ruined thrombin in which the thrombin cleavage site had been mutated to obviate processing were preincubated in the absence (lanes 8 and 12) or presence of 600 nM Ubc5A (lanes 4–10). The GST-RING or GST-RING ruined thrombin in which the thrombin cleavage site had been mutated to obviate processing were preincubated in the absence (lanes 5 and 8) or presence (lanes 6 and 9) of 90 IU/ml of thrombin for 30 min before addition to the conjugation assay. Alternatively, thrombin treatment was conducted after quenching the conjugation reaction (lanes 7 and 10). For both panels, migration positions for relative molecular weight standards are shown to the left. The position of the 5% stacking gel (Stacker), free 14C-ubiquitin, and mono-14C-ubiquitinated RING are shown to the right. C, schematic representation of constructs used in panels A and B.

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TRIM32 Binds Ubc5A via Two Distinct Surfaces on the E2—Ubiquitin carrier proteins bind RING domains such as those present on the TRIM ligases through the Loop 1/2 surface encompassing the N-terminal helix of the E2, as originally shown for UbcH7 binding to Cbl (53). The RING binding interface overlaps that for E1, precluding E1-E2-E3 complexes and requiring dissociation of the E2-ubiquitin thioester intermediate from E1 before binding to the cognate E3 is possible (29, 54). In contrast, we found that truncating the last 10 residues of UbcH5A comprising the C-terminal α-helix, highlighted in yellow in Fig. 6A, abrogates chain formation (Fig. 6B, lane 3 versus 4). Loss of function was not due to disruption of the E2-ubiquitin thioester because Uba1 ternary complex binding occurs at the opposite end of the uncharged E2 (29) and Ubc5AΔ138 supports Uba1-catalyzed transthiolelation (not shown). In addition, the concentration dependence for Ubc5AΔ138 transthiolelation by Uba1 showed wild type kinetics for $K_m$ and $k_{cat}$ (not shown), confirming that the truncation does not obviate correct folding of the protein. Alanine scanning mutagenesis within the 10 truncated residues indicates that some residues have no effect on polyubiquitin chain formation (R139A, E140A, Q143A, K144A, and M147A; Fig. 6B, lanes 7 and 8), whereas other positions qualitatively enhance the rate of chain formation (W141A and T142A; Fig. 6B, lanes 7 and 8). However, mutation of Tyr145 to alanine or Ala146 to a more bulky leucine ablates chain formation (Fig. 6B, lanes 11 and 14). These qualitative results were confirmed by quantitative kinetics for the E2 concentration dependence for $K_m$ and $k_{cat}$.

TRIM32 Binds Ubc5A via Two Distinct Surfaces on the E2—Ubiquitin carrier proteins bind RING domains such as those present on the TRIM ligases through the Loop 1/2 surface encompassing the N-terminal helix of the E2, as originally shown for UbcH7 binding to Cbl (53). The RING binding interface overlaps that for E1, precluding E1-E2-E3 complexes and requiring dissociation of the E2-ubiquitin thioester intermediate from E1 before binding to the cognate E3 is possible (29, 54). In contrast, we found that truncating the last 10 residues of UbcH5A comprising the C-terminal α-helix, highlighted in yellow in Fig. 6A, abrogates chain formation (Fig. 6B, lane 3 versus 4). Loss of function was not due to disruption of the E2-ubiquitin thioester because Uba1 ternary complex binding occurs at the opposite end of the uncharged E2 (29) and Ubc5AΔ138 supports Uba1-catalyzed transthiolelation (not shown). In addition, the concentration dependence for Ubc5AΔ138 transthiolelation by Uba1 showed wild type kinetics for $K_m$ and $k_{cat}$ (not shown), confirming that the truncation does not obviate correct folding of the protein. Alanine scanning mutagenesis within the 10 truncated residues indicates that some residues have no effect on polyubiquitin chain formation (R139A, E140A, Q143A, K144A, and M147A; Fig. 6B, lanes 7 and 8), whereas other positions qualitatively enhance the rate of chain formation (W141A and T142A; Fig. 6B, lanes 7 and 8). However, mutation of Tyr145 to alanine or Ala146 to a more bulky leucine ablates chain formation (Fig. 6B, lanes 11 and 14). These qualitative results were confirmed by quantitative kinetics for the E2 concentration dependence for $K_m$ and $k_{cat}$.

TABLE 1

| $k_{cat}$ | [S]_{0.5} | $n$ |
|---|---|---|
| TRIM32 | 4.5 ± 0.8 × 10^{-3} | 410 ± 10 |
| TRIM25 | 5.2 ± 0.1 × 10^{-2} | 460 ± 20 |
| TRIM50 | 0.28 ± 0.05 × 10^{-3} | 280 ± 43 |
| TRIM22NHIL | 5.6 ± 0.3 × 10^{-3} | 540 ± 12 |
| GST-RING | 26 ± 8 × 10^{-3} | 5000 ± 840 |

$*$ Values of $k_{cat}$ determined as $V_{max}/[E3]_{0.5}$ where [E3]$_{0.5}$ is calculated from total TRIM protein.
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\[ ^{125}\text{I}-\text{ubiquitin} \]

polyubiquitin chain formation determined similar to the study of Fig. 4B and Table 2.

Tyrosine 145 packs between Lys\(^{72}\) and Lys\(^{144}\) (Fig. 6A) so that mutation to alanine would deshield the charge repulsion otherwise present between these residues. Reconstitution of the aromatic nature of the side chain with Ubc5AY145F rescues polyubiquitin chain formation (Fig. 6B, lane 12), consistent with the model for deshielding by the aromatic ring. In contrast, mimicking the phenolate charge by substitution with glutamate does not restore chain formation (Fig. 6B, lane 13). Alanine 146 is buried in Ubc5 and substitution with leucine presumably disrupts packing of the C-terminal helix to the catalytic core of the E2. These results suggest that the C-terminal face of Ubc5A comprises a noncanonical E3 binding surface composed in part of the C-terminal \(\alpha\)-helix and that this interaction requires native packing of this helix.

Although the Ubc5A\(^{138}\) mutant does not support TRIM32-catalyzed chain formation, the data of Fig. 7 indicate that the mutant nonetheless binds TRIM32. The Ubc5A\(^{138}\) mutant, present as its corresponding \(^{125}\text{I}-\text{ubiquitin} \) thioester due to its ability to be charged by Uba1 with wild type kinetics, stimulates Ubc5A-dependent chain formation at concentrations below 100 nM (Fig. 7), consistent with the allosteric cooperativity observed for the wild type E2 (Fig. 4). At higher concentrations, Ubc5A\(^{138}\) displays the same concentration-dependent substrate inhibition observed for wild type protein. The latter is not due to inhibition of the Uba1 step because doubling [Uba1] in parallel control incubations had no effect on rates of chain formation, confirming TRIM32-limiting conditions.

**FIGURE 5.** Glutaraldehyde cross-linking confirms oligomerization of TRIM32\(^{\Delta}\)NHL and GST-RING. Glutaraldehyde cross-linking reactions were conducted with either TRIM32\(^{\Delta}\)NHL (panel A) or GST-RING (panel B) proteins as described under “Materials and Methods” then resolved by 12% (w/v) SDS-PAGE before visualization by Western blotting. Migration positions for relative molecular weight standards are shown to the left.

**FIGURE 6.** Mutations in the C-terminal \(\alpha\)-helix of Ubc5A abrogate polyubiquitin chain formation. A, structure of Ubc5A with the active cysteine shown in orange spheres and the last 10 residues of the protein within the C-terminal \(\alpha\)-helix are highlighted in yellow. The packing of Tyr\(^{145}\) between Lys\(^{72}\) and Lys\(^{144}\) is highlighted. The sequence for the C-terminal 10 residues is shown below the structure. The image was created from PDB file 2C4P (Dodd, R. B., and Read, R. J., unpublished coordinates) using PyMol (Schrodinger). B, autoradiogram comparing wild type Ubc5A (lane 3) and the indicated C-terminal point mutants (lanes 4–15) in their ability to support TRIM32-catalyzed \(^{125}\text{I}-\text{ubiquitin} \) chain formation. Reactions contained 64 nM Uba1, 100 nM active E2, and 200 nM TRIM32 protein, as indicated. Migration positions for relative molecular weight standards are shown to the left. The position of the 5% stacking gel and free \(^{125}\text{I}-\text{ubiquitin} \) are shown to the right.
attached to the target substrate (9). However, the ability of many ubiquitin ligases to assemble free polyubiquitin chains suggests a mechanism in which the chains are first formed then transferred to the target protein (10), as has been shown for the ER-associated ligase gp78 for which chains are assembled on its cognate Ube2g2/Ubc7 E2 carrier protein as a thioester (12). To test this for TRIM32, we exploited the ability of Uba1 to form a Ubc5AC85S-125I-ubiquitin oxyester from the corresponding active site point mutant then purified this inert substrate analog by gel filtration chromatography (33, 55). Such oxyesters are incapable of supporting subsequent E3-catalyzed conjugation reactions due to the stability of the E2-ubiquitin oxyester bond (33). Addition of 5 μM unlabeled ubiquitin to the preformed Ubc5AC85S-125I-ubiquitin oxyester, present at 80 nM, promoted the formation of free polyubiquitin chains (Fig. 8, lane 7) of the expected concentration of free 125I-ubiquitin of 16 nM was significantly below the K_m of 0.8 μM for free ubiquitin shown previously for human Uba1 and predicts a rate of 2% of V_max (33). Addition of 5 μM unlabeled ubiquitin to the incubation of lane 7 reduces the specific radioactivity of the polypeptide to 0.3% of that in lane 6; therefore, high molecular weight conjugates observed in lane 7 must result from conjugation of unlabeled polypeptide to the preformed Ubc5AC85S-125I-ubiquitin oxyester. Determination of associated radioactivity for Ubc5AC85S-125I-ubiquitin oxyester from lanes 6 and 7 show a 0.3 pmol (15%) loss from lane 6 that matched exactly with a 0.3 pmol gain in higher molecular conjugates in lane 7 above the band of the oxyester. In contrast, free ubiquitin-associated radioactivity was unchanged between lanes 6 (0.4 pmol) and 7 (0.4 pmol). Interestingly, TRIM32 is catalytically capable of assembling polyubiquitin chains on Ubc5AΔ138-125I-ubiquitin oxyester (not shown), consistent with the ability of the truncation to bind the ligase (Fig. 7B).

### DISCUSSION

Conjugation of ubiquitin to protein targets is a critical mechanism for cellular regulation in eukaryotes and many of these pathways rely on the assembly of polyubiquitin chains on the targets as the committed step for their degradation by the 26S proteasome or as a scaffold for assembly of multimeric complexes (56, 57). Target protein specificity among these post-translational modifications is defined by a diverse subset of ubiquitin ligases (58) of which the TRIM superfamily constitutes more than 100 paralogs that serve important functions in cellular homeostasis and, more critically, in the innate immune response of cells to viral and bacterial pathogens (13, 14). The current study is the first to investigate the mechanism of polyubiquitin chain formation by TRIM32 and related paralogs, using initial rates for formation of this degradation signal as a functional readout. The results suggest a common catalytic mechanism among members of the superfamily, consistent with their marked conservation in sequence and domain architecture.

The identification of the cognate E2 for a ubiquitin ligase is an essential step in its characterization, yet conservation within the E2 superfamily and the qualitative assays typically used to assess E2 specificity makes this task technically challenging (10). The present biochemically defined quantitative E2 screens unambiguously identify Ubc5 as the sole cognate E2 paralog supporting TRIM32-, TRIM25-, and TRIM5α-catalyzed polyubiquitin chain formation (Fig. 1). The current approach has the advantage that E2 species are compared in parallel at equivalent concentrations determined in functional stoichiometric assays, rather than by total protein, and that activity is assayed under initial velocity conditions so that the signal is proportional to catalytic competence (10). Identification of the ability of the Ubc5 isoforms generally to support TRIM ligase activity agrees with previous observations (36, 59 – 61); however, earlier reports that TRIM32 (36) and TRIM25 (40, 41, 62) also function with UbcH6 likely reflect the intrinsic ability of the E2 to form E3-independent polyubiquitin chains because the protein fails to support the activity of the TRIM paralogs tested here (10). More recently, high throughput methods involving two-hybrid screens have been exploited in an attempt to provide a comprehensive understanding of E2-E3 interactions and have suggested that TRIM32 functions with a broad array of E2 species, including UbcH7 and UbcH8, among others (37). However, such approaches fail to recognize that the actual substrate for an E3 is the cognate E2-ubiquitin thioester, which cannot be replicated in such interaction screens, and that both binding and geometry of the resulting Michaelis complex define catalytic competence. Thus, whereas other uncharged E2 paralogs may bind TRIM32 with low affinity, they can potentially lack the correct orientation to support chain formation as their corresponding ubiquitin thioesters, as appears to be the case for UbcH7 and UbcH8.
Previously we have shown that E2-ubiquitin thioesters bind their cognate ligase with hyperbolic kinetics, from which the intrinsic affinity \(K_d\) could be determined from the corresponding \(K_m\) (10, 30, 33). In contrast, the TRIM ligases exhibit cooperative allosteric kinetics with respect to Ubc5-ubiquitin thioester concentration, requiring binding-dependent conformational cross-talk among the subunits of the ligase (Fig. 4). This is the first evidence of cooperativity for a ubiquitin ligase and provides important insights into the mechanism of polyubiquitin chain formation because deletion of the B box-coiled coil oligomerization domain abrogates chain formation unless association of the catalytic RING domains is rescued by linkage to an artificial dimerization domain such as GST (Fig. 3 and Table 1). Similar rescue of activity through fusion to GST has been reported previously for the RING domains of c-Cbl (63), APC11 (64, 65), BRCA1 (66), ROC1 and -2 (67), and Bcr-Abl protein (34), although the mechanism by which the GST moiety restores activity was not pursued. Mounting evidence suggests homo- and heterodimerization is required for the activity of many ligases (68–72) (reviewed in Ref. 73). The current kinetic studies suggest a mechanistic context for oligomerization as a necessary component of polyubiquitin chain formation.

The current kinetic data require that conformational communication among the multiple E2-ubiquitin binding sites occurs across the oligomer and that the portion of TRIM32 from the N terminus to the B-box mediates this mechanical coupling, evidenced by the loss of cooperativity in favor of hyperbolic kinetics with respect to \([Ubc5A_{\Delta 138}]_0\) for the isolated RING-catalyzed automonoubiquitination (Fig. 4, C and D). From the theoretical sigmoidal curve we can calculate the Hill coefficient \(n\), a direct measure of the lower limit to the number of substrate binding sites (74). The range of \(n\) values for TRIM32 and TRIM32\(\Delta\)NHIL indicate that there are several binding sites on the E3; however, limitations imposed by substrate inhibition at higher concentrations of the Ubc5-ubiquitin thioester precludes accurate estimates of the intrinsic Hill coefficient. That the GST-RING displays a lower \(n\) value may indicate a diminished number of substrate binding sites, but more likely results from ablated conformational coupling of the subunits when linked artificially through GST. Despite this limita-
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The data indicate that there are at least four Ubc5-ubiquitin thioester binding sites present on wild type TRIM32. Furthermore, an n value equal to two for GST-RING requires more than two binding sites for Ubc5-ubiquitin thioester, recapitulating the four binding sites of the wild type protein, but with diminished conformational coupling.

Nonlinear regression analysis allows calculation of values for [S]_{1/2} that are comparable (but not formally equivalent) to the \( K_m \) for hyperbolic enzymes. The range of [S]_{1/2} values for the TRIM paralogs examined are somewhat higher than the \( K_m \) of 54 ± 18 nm previously reported for the interaction between human Ub2h-ubiquitin thioester with E3a/Ubr1 (33). Values of \( K_m \) frequently evolve to approximate the corresponding intracellular substrate concentration so that rates of the enzyme fluctuate with changes in metabolite concentration. Using quantitative Western blots, we estimate the intracellular concentration of Ubc5 in A549 cells to be ~ 160 nm, somewhat lower than the [S]_{1/2} values for the three TRIM ligases tested (Table 1). However, because of the sharp increase in activity inherent in the sigmoidal cooperative concentration dependence observed here, small changes in Ubc5 concentration will result in large alterations in activity, rendering such enzymes molecular switches. In the case of the TRIM ligases, the observed substrate inhibition at higher concentrations of Ubc5-ubiquitin thioester further enforces a narrow range of activity.

Substrate inhibition at high E2 concentrations was previously observed with the E3α/Ubr1 RING ligase and the Hect ligase family (10, 33). In the present context, substrate inhibition is consistent with ordered binding of the Ubc5-ubiquitin thioester to distinct substrate sites on the TRIM ligases that differ in affinity. Previous structural work demonstrates that E2 proteins bind the RING domain through a specific surface near their N terminus encompassing the Loop 1/2 region (53). However, the current study suggests that TRIM32 also binds Ubc5-ubiquitin thioester independently through an additional non-canonical surface on the C-terminal α-helix because truncation of the last 10 Ubc5A residues abrogates free chain formation and this effect can be replicated by mutation of Tyr^{145} (Fig. 6 and Table 2). However, because the Ubc5A138 mutant can stimulate the rate of polyubiquitin chain formation through allosteric cooperativity even though it cannot formally support the net reaction of chain elongation demonstrates that the substrate is capable of binding to TRIM32, presumably through the canonical RING binding interaction.

Overall, the current data support a model for TRIM32 and other members of this ligase superfamily in which oligomerization is essential for catalytic competence in formation of the resulting polyubiquitin degradation signal. Other observations suggest that polyubiquitin is first assembled as an enzyme-bound E2 thioester intermediate prior to transfer en bloc to the protein target, although the latter step has not been directly confirmed. Such a mechanism obviates the topological constraints posed by translating a catalytic site along the growing polyubiquitin chain, whereas target protein remains bound. Empirical evidence for distinct TRIM32 binding sites on E2, substrate inhibition by the corresponding E2-ubiquitin thioester, and the stoichiometry of the Hill coefficient relative to the oligomerization state of the ligase are consistent with multiple binding sites per subunit for the enzyme. Whether this mechanism conforms to a “seesaw” model proposed earlier (9) or an indexation model in which chains are built “inside out” remains to be determined. However, ordered E2-ubiquitin thioester binding to distinct nonsymmetric sites, indicated by substrate inhibition at higher concentrations, strongly favors the latter alternative. Nonetheless, the present observations provide the first detailed examination of the mechanism of TRIM ligase function.

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REFERENCES

1. Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989) A mult ubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science 243, 1576–1583
2. Pickart, C. M. (2001) Mechanism underlying ubiquitination. Annu. Rev. Biochem. 70, 503–533
3. Ravid, T., and Hochstrasser, M. (2008) Diversity of degradation signals in the ubiquitin-proteasome system. Nat. Rev. Mol. Cell Biol. 9, 679–690
4. Deshaies, R. J., and Joazeiro, C. A. (2009) RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 78, 399–434
5. Haas, A. L., and Siepmann, T. J. (1997) Pathways of ubiquitin conjugation. FASEB J. 11, 1257–1268
6. Streich, F. C., Jr., and Haas, A. L. (2010) Activation of ubiquitin and ubiquitin-like proteins. Subcell. Biochem. 54, 1–16
7. Pickart, C. M., and Rose, I. A. (1985) Functional heterogeneity of ubiquitin carrier proteins. J. Biol. Chem. 260, 1573–1581
8. Ye, Y., and Rape, M. (2009) Building ubiquitin chains. E2 enzymes at work. Nat. Rev. Mol. Cell Biol. 10, 755–764
9. Hochstrasser, M. (2006) Linking mysteries of ubiquitin-chain assembly. Cell 124, 27–34
10. Ronchi, V. P., and Haas, A. L. (2012) Measuring rates of ubiquitin chain formation as a functional readout of ligase activity. Meth. Mol. Biol. 832, 197–218
11. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. Proc. Natl. Acad. Sci. USA. 96, 11364–11369
12. Li, W., Tu, D., Brunger, A. T., and Ye, Y. (2007) A ubiquitin ligase transfers preformed polyubiquitin chains from a conjugating enzyme to a substrate. Nature 446, 333–337
13. Nisole, S., Stoye, J. P., and Sahb, A. (2005) TRIM family proteins. Retroviral restriction and antiviral defence. Nat. Rev. Microbiol. 3, 799–808
14. McNab, F. W., Rajsbaum, R., Stoye, J. P., and O’Garra, A. (2011) Tripartite-motif proteins and innate immune regulation. Curr. Opin. Immunol. 23, 46–56
15. Munir, M. (2010) TRIM proteins. Another class of viral victims. Sci. Signal. 3, jc2
16. Horn, E. J., Albor, A., Liu, Y., El-Hizawi, S., Vanderbeek, G. E., Babcock, M., Bowden, G. T., Hennings, H., Lozano, G., Weinberg, W. C., and Kulesz-Martin, M. (2004) RING protein Trim32 associated with skin carcinogenesis has anti-apoptotic and E3-ubiquitin ligase properties. Carcinogenesis 25, 157–167
17. Albor, A., and Kulesz-Martin, M. (2007) Novel initiation genes in squamous cell carcinogenesis. A role for substrate-specific ubiquitylation in the control of cell survival. Mol. Carcinog. 46, 585–590
18. Kano, S., Miyajima, N., Fukuda, S., and Hatakeyama, S. (2008) Tripartite motif protein 32 facilitates cell growth and migration via degradation of Abl-interactor 2. Cancer Res. 68, 5572–5580
Mechanism of TRIM-catalyzed Chain Formation

19. Liu, Y., Lagowski, J. P., Gao, S., Raymond, J. H., White, C. R., and Kulesz-Martin, M. (2010) Regulation of the psoriatic chemokine CCL20 by E3 ligases TRIM32 and Piaxy in keratinocytes. J. Invest Dermatol. 130, 1384–1390

20. Yokota, T., Mishra, M., Akatsu, H., Tani, Y., Miyauchi, T., Yamamoto, T., Kosaka, K., Nagai, Y., Sawada, T., and Heese, K. (2006) Brain site-specific gene expression analysis in Alzheimer’s disease patients. Eur. J. Clin. Invest. 36, 820–830

21. Kudryashova, E., Wu, J., Havton, L. A., and Spencer, M. J. (2009) Deficiency of the E3 ubiquitin ligase TRIM32 in mice leads to a myopathy with a neurogenic component. Hum. Mol. Genet. 18, 1353–1367

22. Schwamborn, J. C., Berezikov, E., and Knoblich, J. A. (2009) The TRIM-NHL protein TRIM32 activates microRNAs and prevents self-renewal in mouse neural progenitors. Cell 136, 913–925

23. Guglielmi, M., Magri, F., and Comi, G. P. (2005) Molecular etiopathogenesis of limb girdle muscular and congenital muscular dystrophies. Boundaries and contingencies. Clin. Chim. Acta 361, 54–79

24. Muller, J., Stoettzel, C., Vincent, M. C., Leitch, C. C., Laurier, V., Danse, J. M., Helle, S., Marion, V., Bennouna-Greene, V., Vicaire, S., Megarbane, A., Kaplan, I., Drouin-Garraud, V., Hamdani, M., Signaud, S., Franconnet, C., Roume, J., Bitoun, P., Goldenberg, A., Philip, N., Odent, S., Green, J., Cossée, M., Davis, E. E., Katsanis, N., Bonneau, D., Verloes, A., A., Akira, S., Chen, Z., Inoue, S., and Jung, J. U. (2007) TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature 446, 916–920

25. Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., and Chen, Z. J. (2010) Reconstitution of the RIG-1 pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell 141, 315–320

26. Wilkinson, K. D., Tashayev, V. L., O’Connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995) Metabolism of the polyubiquitin degradation signal. Structure, mechanism, and role of isopeptide T. Biochemistry 34, 14535–14546

27. Haas, A. L. (2005) Purification of E1 and E1-like enzymes. Methods Mol. Biol. 301, 23–35

28. Haas, A. L., and Bright, P. M. (1988) The resolution and characterization of putative ubiquitin carrier protein isozymes from rabbit reticulocytes. J. Biol. Chem. 263, 13258–13267

29. Tokgoz, Z., Siepmann, T. J., Streich, F., Jr., Kumar, B., Klein, J. M., and Haas, A. L. (2012) E1-E2 interactions in the ubiquitin and Nedd8 ligation pathways. J. Biol. Chem. 287, 311–321

30. Kumar, B., Lecompte, K. G., Klein, J. M., and Haas, A. L. (2010) Ser120 of Ubc2/Rad6 regulates ubiquitin-dependent N-end rule targeting by E3a/Ubr1. J. Biol. Chem. 285, 41300–41309

31. Russell, N. S., and Wilkinson, K. D. (2005) Deubiquitinating enzyme purification, assay inhibitors, and characterization. Methods Mol. Biol. 301, 207–219

32. Haas, A. L., and Rose, I. A. (1981) Hemin inhibits ATP-dependent ubiquitin-dependent proteolysis. Role of hemin in regulating ubiquitin conjugate degradation. Proc. Natl. Acad. Sci. U.S.A. 77, 1365–1368

33. Siepmann, T. J., Bohnsack, R. N., Tokgoz, Z., Baboshina, O. V., and Haas, A. L. (2003) Protein interactions within the N-end rule ubiquitin ligation pathway. J. Biol. Chem. 278, 9494–9457

34. Maru, Y., Afar, D. E., Witte, O. N., and Shibuya, M. (1996) The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain. J. Biol. Chem. 271, 15353–15357

35. Wimm, P. J., Belga, T. L., Battey, J. N., Barnerjee, A., and Wade, R. (2004) Determinants of functionality in the ubiquitin conjugating enzyme family. Structure 12, 1563–1574

36. Kudryashova, E., Kudryashov, D., Kramerova, I., and Spencer, M. J. (2005) Trim32 is a ubiquitin ligase mutated in limb girdle muscular dystrophy type 2H that binds to skeletal muscle myosin and ubiquitinates actin. J. Biol. Mol. 354, 413–424

37. Napolitano, L. M., Jaffray, E. G., Hay, R. T., and Meroni, G. (2011) Functional interactions between ubiquitin E2 enzymes and TRIM proteins. Biochem. J. 434, 309–319

38. Hershko, A., and Heller, H. (1985) Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. Biochem. Biophys. Res. Commun. 128, 1079–1086

39. Hershko, A., and Heller, H. (1985) Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. Biochem. Biophys. Res. Commun. 130, 1384–1390

40. Urano, T., Saito, T., Tsukui, T., Fujita, M., Hosoi, T., Muramatsu, M., Ouchi, Y., and Inoue, S. (2002) Efp targets 14-3-3 to regulate the ubiquitin system via proteolytic and non-proteolytic mechanisms. J. Biol. Chem. 278, 6845–6848

41. Ouchi, Y., and Inoue, S. (2002) Efp targets 14-3-3 to regulate the ubiquitin system via proteolytic and non-proteolytic mechanisms. J. Biol. Chem. 278, 6845–6848

42. Martin, M. F. (2010) Regulation of the psoriatic chemokine CCL20 by E3 ubiquitin ligases. Self-association of ubiquitin is regulated by the ubiquitin carboxy-terminal hydrolase UCH37 in keratinocytes. J. Biol. Chem. 285, 41300–41309

43. Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., and Chen, Z. J. (2010) Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell 141, 315–320

44. Vargo, M. A., Nguyen, L., and Colman, R. F. (2004) Subunit interface residues of glutathione S-transferase A1-1 that are important in the monomer-dimer equilbrium. Biochemistry 43, 3327–3335

45. Li, X., Zhang, P., Armstrong, R. N., and Gilliland, G. L. (1992) The three-dimensional structure of a glutathione S-transferase from the mu gene class. Structural analysis of the binary complex of isozyme 3-3 and glutathione at 2.2-Å resolution. Biochemistry 31, 10169–10184

46. Baboshina, O. V., Crinelli, R., Siepmann, T. J., and Haas, A. L. (2001) N-end rule specificity within the ubiquitin/proteasome pathway is not an affinity effect. J. Biol. Chem. 276, 39428–39437

47. Vargo, M. A., Nguyen, L., and Colman, R. F. (2004) Subunit interface residues of glutathione S-transferase A1-1 that are important in the monomer-dimer equilbrium. Biochemistry 43, 3327–3335

48. Zhang, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) Structure of a c-Chi-UbcH7 complex. RING domain function in ubiquitin-protein ligases. Cell 102, 533–539

49. Eletr, Z. M., Huang, D. T., Duda, D. M., Schulman, B. A., and Kuhlman, B. (2005) E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. Nat. Struct. Mol. Biol. 12, 933–943

50. Sullivan, M. D., and Vierstra, R. D. (1993) Formation of a stable adduct between ubiquitin and the Arabidopsis ubiquitin-conjugating enzyme, AtUBC1. J. Biol. Chem. 268, 8777–8780

51. Ikeda, F., and Dikic, I. (2008) Atypical ubiquitin chains. New molecular signals. EMBO Rep. 9, 536–542

52. Kim, I., and Rao, H. (2006) What’s Ub chain linkage got to do with it? Sci. STKE 2006, e18

53. de Bie, P., and Ciechanover, A. (2011) Ubiquitination of E3 ligases. Self-regulation of the ubiquitin system via proteolytic and non-proteolytic mechanisms. Cell Death. Differ. 18, 1393–1402
Yamauchi, K., Wada, K., Tanji, K., Tanaka, M., and Kamitani, T. (2008) Ubiquitination of E3 ubiquitin ligase TRIM5α and its potential role. FEBS J. 275, 1540–1555

Urano, T., Usui, T., Takeda, S., Ikeda, K., Okada, A., Ishida, Y., Iwayanagi, T., Otomo, J., Ouchi, Y., and Inoue, S. (2009) TRIM44 interacts with and stabilizes terf, a TRIM ubiquitin E3 ligase. Biochem. Biophys. Res. Commun. 383, 263–268

Chen, D., Gould, C., Garza, R., Gao, T., Hampton, R. Y., and Newton, A. C. (2007) Amplitude control of protein kinase C by RINCK, a novel E3 ubiquitin ligase. J. Biol. Chem. 282, 33776–33787

Horie-Inoue, K., and Inoue, S. (2006) Epigenetic and proteolytic inactivation of 14-3-3ζ in breast and prostate cancers. Semin. Cancer Biol. 16, 235–239

Joazeiro, C. A., Wing, S. S., Huang, H., Levenson, J. D., Hunter, T., and Liu, Y. C. (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science 286, 309–312

Levenson, J. D., Joazeiro, C. A., Page, A. M., Huang, H., Hieter, P., and Hunter, T. (2000) The APC11 RING-H2 finger mediates E2-dependent ubiquitination. Mol. Biol. Cell 11, 2315–2325

Gmachl, M., Gieffers, C., Podtelejnikov, A. V., Mann, M., and Peters, J. M. (2000) The RING-H2 finger protein APC11 and the E2 enzyme UBC4 are sufficient to ubiquitinate substrates of the anaphase-promoting complex. Proc. Natl. Acad. Sci. U.S.A. 97, 8973–8978

Ruffner, H., Joazeiro, C. A., Hemmati, D., Hunter, T., and Verma, I. M. (2001) Cancer-predisposing mutations within the RING domain of BRCA1. Loss of ubiquitin protein ligase activity and protection from radiation hypersensitivity. Proc. Natl. Acad. Sci. U.S.A. 98, 5134–5139

Furukawa, M., Ohta, T., and Xiong, Y. (2002) Activation of UBC5 ubiquitin-conjugating enzyme by the RING finger of ROC1 and assembly of active ubiquitin ligases by all cullins. J. Biol. Chem. 277, 15758–15765

Barbash, O., Zamfirova, P., Lin, D. I., Chen, X., Yang, K., Nakagawa, H., Lu, F., Rustgi, A. K., and Diehl, J. A. (2008) Mutations in Fbx4 inhibit dimerization of the SCF^{Fbx4} ligase and contribute to cyclin D1 overexpression in human cancer. Cancer Cell 14, 68–78

Brzovic, P. S., Rajagopal, P., Hoyt, D. W., King, M. C., and Klevit, R. E. (2001) Structure of a BRCA1-BA pierced RING-RING complex. Nat. Struct. Biol. 8, 833–837

Buchwald, G., van der Stoop, P., Weichenrieder, O., Perrakis, A., van Loenhoven, M., and Sixma, T. K. (2006) Structure and E3-ligase activity of the Ring-Ring complex of Polycomb proteins Bmi1 and Ring1b. EMBO J. 25, 2465–2474

Linke, K., Mace, P. D., Smith, C. A., Vaux, D. L., Silke, J., and Day, C. L. (2008) Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. Cell Death Differ. 15, 841–848

Mace, P. D., Linke, K., Feltham, R., Schumacher, F. R., Smith, C. A., Vaux, D. L., Silke, J., and Day, C. L. (2008) Structures of the cIAP2 RING domain reveal conformational changes associated with ubiquitin-conjugating enzyme (E2) recruitment. J. Biol. Chem. 283, 31633–31640

Knipscheer, P., and Sixma, T. K. (2007) Protein-protein interactions regulate Ubl conjugation. Curr. Opin. Struct. Biol. 17, 665–673

Segal, I. A. (1975) Enzyme Kinetics, Wiley-Interscience, New York