The ubiquitin fusion degradation (UFD) pathway is a proteolytic system conserved in yeast and mammals, in which an uncleavable ubiquitin moiety linked to the N terminus of a protein functions as a degradation signal of the fusion protein. Although key components of the UFD pathway in yeast have been identified, the E3 enzyme of the human UFD pathway has not been studied. In this work, we show that TRIP12 is the E3 enzyme of the human UFD pathway. Thus, TRIP12 catalyzes in vitro ubiquitination of UFD substrates in conjunction with E1, E2, and E4 enzymes. Knockdown of TRIP12 stabilizes not only artificial UFD substrates but a physiological substrate UBB\textsuperscript{+1}. Moreover, TRIP12 knockdown reduces UBB\textsuperscript{+1}-induced cell death in human neuroblastoma cells. Surprisingly, complementation of TRIP12 knockdown cells with the TRIP12 HECT domain mostly restores efficient degradation of UFD substrates, indicating that the TRIP12 HECT domain can act as the E3 enzyme for the UFD pathway in human cells. The TRIP12 HECT domain directs ubiquitination of UFD substrates in vitro and can be specifically cross-linked to the ubiquitin moiety of the substrates in vivo, suggesting that the TRIP12 HECT domain possesses a noncovalent ubiquitin-binding site. In addition, we demonstrate that Ub\textsuperscript{ΔGG}, a mutant ubiquitin that cannot be conjugated to other proteins, is a substrate of the TRIP12 HECT domain both in vivo and in vitro, indicating that the C-terminal extension fused to the uncleavable ubiquitin is not required for substrate recognition in the UFD pathway. These results provide new insights into the mechanism of the mammalian UFD pathway and the functional nonequivalence of different HECT domains.

Ubiquitin-dependent proteolysis plays an important role in various cellular events, such as cell cycle regulation and signal transduction (1). The covalent conjugation of ubiquitin to a target protein involves an enzyme cascade. The ubiquitin-activating enzyme (E1)\textsuperscript{2} activates ubiquitin in an ATP-dependent reaction by forming a thioester bond with the C-terminal gly- cine of ubiquitin. The ubiquitin is then transferred to a specific sulphydryl group on a ubiquitin-conjugating enzyme (E2). A ubiquitin-protein ligase (E3) transfers the activated ubiquitin from E2 to a lysine residue of a bound substrate, forming an isopeptide bond. Substrate specificity is determined primarily by E3, which binds both the protein substrate and the cognate E2. Once the mult ubiquitin chain is assembled on a protein substrate, the target protein is recognized and degraded by the 26S proteasome (1–3).

E3 enzymes are classified into two main families: HECT (homologous to E6AP C terminus) domain E3s and RING (really interesting new gene) domain E3s (2). Members of the HECT family contain a diverse N-terminal region and a conserved C-terminal HECT domain, which has a reactive Cys residue. HECT E3s form a thioester intermediate with ubiquitin at the conserved Cys residue before transfer of ubiquitin to substrates (4), whereas RING E3s do not form a covalent bond with ubiquitin. Recent studies have shown that different HECT E3s utilize distinct mechanisms for the synthesis of the polyubiquitin chain (5). Thus, E6AP assembles a ubiquitin chain on the Cys residue of the HECT domain, whereas KIAA10 builds a ubiquitin chain as a free entity.

The ubiquitin fusion degradation (UFD) pathway is a proteolytic system in which an uncleavable ubiquitin moiety linked to the N terminus of a protein functions as a degradation signal for the fusion protein (6). Genetic and biochemical studies have defined key components of the UFD pathway in Saccharomyces cerevisiae (6, 7). Ubiquitination of UFD substrates is initiated by the HECT domain E3 UFD4 in conjunction with E1 and E2. Pol yubiquitin chain formation additionally requires UFD2, which extends ubiquitin chains on UFD substrates. Although the UFD pathway is conserved in yeast and mammalian cells (8), the human E3 has not been characterized.

Sequence similarity search with yeast UFD4 against a protein sequence database picks up TRIP12 (thyroid hormone-interacting protein 12) as a probable human homologue of yeast UFD4, with the highest similarity occurring in the HECT domain. In this study, we showed that TRIP12 is the E3 enzyme responsible for the UFD pathway in human cells. We also demonstrated that the HECT domain of TRIP12 ubiquitinates UFD substrates by recognizing the ubiquitin moiety of the substrates.
and that the C-terminal extension fused to the uncleavable ubiquitin is not required for recognition. Our data indicate that the TRIP12 HECT domain is unique in that the HECT domain can conjugate ubiquitin to noncovalently bound ubiquitin.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Full-length human TRIP12 cDNA (accession number D28476) and UFD2a cDNA (NM_006048) were obtained from the Kazusa DNA Research Institute and subcloned into pCDNA3.1 (Invitrogen) or pYR vectors (9). Full-length human E6AP cDNA (BC002582) was obtained from Incyte Genomics Inc. DNA fragments corresponding to the TRIP12 HECT domain (residues 1602–2003) and the E6AP HECT domain (residues 495–852) were amplified by PCR with appropriate primers and subcloned into expression vectors. The TRIP12 HECT C1972S mutant with a cysteine to serine substitution at the conserved cysteine was generated by introducing point mutations using the QuikChange kit (Stratagene). The ubiquitination assay was performed in a 20-μl reaction containing 25 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM ATP, 10 mM MgCl2, 1 mM dithiothreitol, 800 ng of ubiquitin, 100 ng of Uba1, 250 ng of UbcH5a, 300 ng of UbG76V-GFP, and the indicated amounts of TRIP12 or E6AP. Reaction mixtures were incubated at 37 °C for 1 h, terminated by the addition of 20 μl of 2 X Laemmli sample buffer, and resolved by SDS-PAGE followed by Western blot analysis. In Vivo Cross-linking with Dithiobis(succinimidylpropionate) (DSP)—Transfected cells were washed with PBS (137 mM NaCl, 0.67 mM KCl, 8 mM Na2HPO4, 1.4 mM KH2PO4) three times, and DSP (Pierce) dissolved in DMSO was added to a final concentration of 1 mM. Cells were gently swirled for 30 min at room temperature and the reaction was stopped by the addition of Tris-Cl (pH 7.4) to a final concentration of 50 mM. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM dithiothreitol, 0.5% (v/v) Triton X-100, 10% (v/v) glycerol, 5 μg/ml leupeptin, 5 μg/ml antipain, 5 μg/ml pepstatin A, and 5 μg/ml aprotinin. Lysates were clarified by centrifugation at 10,000 × g for 20 min at 4 °C. FLAG-tagged proteins were purified by applying the supernatant onto a FLAG-M2-agarose column (Sigma) equilibrated with the lysis buffer. After extensive washing of the column with the lysis buffer, the bound proteins were eluted with the lysis buffer containing 0.3 mg/ml FLAG peptide (Sigma).

**In Vivo Cross-linking with Dithiobis(succinimidylpropionate) (DSP)—**Transfected cells were washed with PBS (137 mM NaCl, 0.67 mM KCl, 8 mM Na2HPO4, 1.4 mM KH2PO4) three times, and DSP (Pierce) dissolved in DMSO was added to a final concentration of 1 mM. Cells were gently swirled for 30 min at room temperature and the reaction was stopped by the addition of Tris-Cl (pH 7.4) to a final concentration of 50 mM. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, and 1.0% Nonidet P-40. Cell lysates were adjusted to 0.1% Nonidet P-40. Cell lysates were adjusted to 0.1% Nonidet P-40. Cell lysates were adjusted to 0.1% Nonidet P-40.

**Ubiquitination of UFD Substrates by the TRIP12 HECT Domain**

**Transfection and Western Blotting**—Transfection was performed using the polyethyleneimine methodology (10). After 36 h, cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethanesulfonyl fluoride, and 1.0% Nonidet P-40. Proteins in cell lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Whatman), and visualized by Western blotting with enhanced chemiluminescence reagents (Amersham Biosciences). For Western blotting, we purchased commercial antibodies against FLAG (Sigma), GFP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), GAPDH (Chemicon), and GST (Santa Cruz Biotechnology).

**RNA Interference**—The TRIP12-specific siRNA oligonucleotides (TRIP12-1 for the target sequence 5′-GCA CUU CAG CAU ACU GAA U-3′ and TRIP12-2 for the sequence 5′-AGU ACU CAC CUC CAA GAG A-3′) and control scrambled siRNA oligonucleotides (5′-CCU ACG CCA CCA AUU UGG U-3′) were chemically synthesized by Bioneer (Daejeon, Korea). Cells were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen and knockdown). After 72 h, the cells were harvested and knockdown of target genes was assessed by Western blotting.

**Lentivirus-mediated TRIP12 RNA Interference**—To establish HeLa cell lines with stable knockdown of TRIP12, a set of pLKO.1 vectors containing stem-loop cassettes encoding shRNAs targeted to human TRIP12 (TRCN 22374, TRCN 22375, TRCN 22376, TRCN 22377, and TRCN 22378) and pLKO.1-puro control vector, which does not contain an shRNA insert, were purchased from Sigma (MISSION TRC-Hs 1.0 shRNA library). Vectors were expanded in competent Esche- richia coli (TOP10 cells; Invitrogen) and purified using the Maxiprep kit (Qiagen). To generate lentiviral particles, human embryonic kidney 293 cells were cotransfected with the lentiviral vector along with a compatible packaging plasmid mixture (Virapower lentiviral packaging system; Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. HeLa cells were exposed to lentivirus-containing supernatant for 16 h in the presence of 6 μg/ml Polybrene (Sigma). After infection, cells were selected in the presence of puromycin (2 μg/ml; Invitrogen) for 2 weeks. Stable transfectants were tested for suppression of TRIP12 expression. The two cell lines with the most efficient knockdown were chosen for subsequent experiments.
Cell Viability Assay and Hoechst Staining for Apoptosis Quantification—Cell viability was assessed by a colorimetric procedure with Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Equal numbers of SH-SY5Y cells (10^4 cells/well) were seeded into a 96-well plate. Twenty-four hours after seeding, cells were transfected with siRNA duplexes using Lipofectamine 2000 (Invitrogen). After further incubation for 48 h, the expression levels of reporters were determined by Western blotting with anti-GFP antibody. For Hoechst staining, cells were fixed with 3.4% paraformaldehyde in PBS for 10 min. After washing twice with PBS, cells were stained for 2 min with Hoechst 33258 (20 ng/ml in PBS). Cells were harvested at the time points shown, and the expression levels of reporters were determined by Western blotting with anti-GFP antibody. In all cases, GAPDH levels were measured as a loading control. The results were subjected to statistical analysis.

RESULTS AND DISCUSSION

Knockdown of TRIP12 Stabilizes UFD Substrates—To investigate the role of TRIP12 in the UFD pathway in human cells, expression of TRIP12 was transiently blocked by transfection of TRIP12-specific siRNAs into HeLa cells, and the stability of the UFD substrate was evaluated. The efficacy of siRNA-mediated TRIP12 knockdown was determined by Western blot analysis following sequential transfection of TRIP12 siRNAs and

FIGURE 1. Knockdown of TRIP12 stabilizes the UFD substrate. A, siRNA-mediated suppression of FLAG-tagged TRIP12 expression. HeLa cells were transfected with scrambled or TRIP12-specific siRNAs, as indicated. After 12 h, siRNA-treated cells were transfected with FLAG-tagged TRIP12. To evaluate the knockdown efficiency of siRNA-treated cells, cell lysates were analyzed by Western blotting (WB) with anti-FLAG antibody. B, expression of GFP-based reporters with a degradation signal in TRIP12 knockdown cells. HeLa cells were transfected with scrambled or TRIP12-specific siRNA as indicated. Twelve hours later, the expression constructs for GFP-based reporters were transfected into siRNA-treated cells. After further incubation for 48 h, the expression levels of reporters were determined by Western blotting with anti-GFP antibody. C, stabilization of the UFD substrate in TRIP12 knockdown cells. HeLa cells were engineered to stably express either an shRNA directed against TRIP12 (shTRIP12-1) or a scrambled shRNA (shControl) and transfected with the expression construct for GFP-based reporters. Thirty-six hours after transfection, cycloheximide was added to all samples. Cells were harvested at the time points shown, and the expression levels of reporters were determined by Western blotting with anti-GFP antibody. In all cases, GAPDH levels were measured as a loading control. The graphs in the right panels illustrate densitometric analysis of GFP signals. All experiments were repeated at least three times. Similar results were obtained with another TRIP12 knockdown cell line, shTRIP12-2. D, siRNA-mediated knockdown of human UFD2a and human UFD2b expression. HeLa cells were transfected with human UFD2a, human UFD2b, or control scrambled siRNAs, as indicated. After 60 h, total cellular RNA was isolated and subjected to real-time reverse transcription-PCR analysis using specific primers for human UFD2a, human UFD2b, and GAPDH mRNA. The relative amounts of human UFD2a and human UFD2b mRNAs were normalized to GAPDH values and expressed relative to the control siRNA-treated samples. E, stabilization of the UFD substrate by knockdown of human UFD2a and human UFD2b. HeLa cells were transfected with scrambled or human UFD2a- and human UFD2b-specific siRNAs, as indicated. Transfection and Western blotting of UbG76V-GFP were performed as described in Fig. 1B. Thirty-six hours after transfection, cycloheximide was added to all samples. GAPDH levels were used as a loading control.
the TRIP12 expression construct. As shown in Fig. 1A, expression of FLAG-TRIP12 was greatly reduced by TRIP12-specific siRNAs but not by scrambled siRNA, indicating that depletion of TRIP12 by siRNA was efficient and specific. We then examined whether the expression of UbG76V-GFP and Ub-P-GFP, model substrates of the UFD pathway (8), was affected when TRIP12 expression was knocked down. The expression levels of UbG76V-GFP and Ub-P-GFP were much higher in TRIP12 knockdown cells than in control knockdown cells (Fig. 1B). In contrast, the expression of GFP or Ub-R-GFP, a well-defined N-end rule substrate (8), was not affected by knockdown of TRIP12 (Fig. 1B), suggesting that TRIP12 was specifically involved in the UFD pathway. To examine whether the protein stability of the UFD substrate was affected by TRIP12 knockdown, we generated HeLa cell lines with stable knockdown, we generated HeLa cell lines with stable knockdown of TRIP12. UbG76V-GFP was expressed in control and TRIP12 knockdown cells, and its levels were monitored after inhibiting new protein synthesis with cycloheximide. As shown in Fig. 1C, UbG76V-GFP was significantly stabilized in TRIP12 knockdown cells compared with control knockdown cells, whereas the stability of Ub-R-GFP was not affected by TRIP12 knockdown. Taken together with the sequence homology of TRIP12 to UFD4, these data suggest that TRIP12 is the human E3 ubiquitin ligase of the UFD pathway.

The human genome contains two genes, UFD2a and UFD2b, homologous to yeast UFD2, the E4 enzyme in yeast UFD pathway. To examine whether human UFD2a and human UFD2b are involved in the UFD pathway in human cells, their expression was knocked down by siRNA treatment, and the protein stability of the UFD substrate was analyzed. Quantitative real-time PCR analysis of human UFD2a and human UFD2b expression following siRNA treatments revealed that human UFD2a and human UFD2b mRNA levels were greatly reduced by human UFD2-specific siRNAs (Fig. 1D). As shown in Fig. 1E, knockdown of human UFD2a and human UFD2b significantly stabilized UbG76V-GFP, suggesting that E4 activity is also required in human UFD pathway.

The TRIP12 HECT Domain Can Reverse Suppression of the UFD Pathway in TRIP12 Knockdown Cells—In one of the stable knockdown cell lines (named shTRIP12-1), short hairpin RNA was targeted against the 3′-untranslated region of TRIP12 mRNA. In shTRIP12-1 cells, endogenous TRIP12 expression was suppressed, but TRIP12 expression derived from transfected plasmids was not affected, since the expression plasmids did not contain the untranslated region. To confirm that the increase in UbG76V-GFP expression in TRIP12 knockdown cells was due to the suppression of TRIP12 expression, we transfected shTRIP12-1 cells with a TRIP12 expression construct. Expression of TRIP12 efficiently lowered UbG76V-GFP expression in a dose-dependent fashion (Fig. 2A), indicating that knockdown of TRIP12 expression was responsible for the increased UbG76V-GFP level. To show that TRIP12-mediated degradation of UbG76V-GFP was dependent on the proteasome activity, TRIP12 complementation experiments with shTRIP12-1 cells were performed in the presence of proteasome inhibitor MG132. The inhibition of proteasome by MG132 greatly enhanced the UbG76V-GFP level not only in control cells but in shTRIP12-1 cells complemented with TRIP12 (Fig. 2A), indicating that TRIP12-dependent UbG76V-GFP degradation requires the proteasome activity.

In HECT domain E3s, the C-terminal HECT domain interacts with charged E2, whereas the N-terminal portion recognizes the substrate. To address the function of the N-terminal part of TRIP12 in UFD substrate recognition, we transfected TRIP12 knockdown cells with a construct designed to express the TRIP12 HECT domain only. Remarkably, the level of UbG76V-GFP was decreased by the TRIP12 HECT domain in a dose-dependent manner (Fig. 2B). In contrast, the E6AP HECT domain or TRIP12 HECT domain with a cysteine to serine mutation at the conserved cysteine did not alter UbG76V-GFP expression (Fig. 2B), indicating that the catalytically active HECT domain of TRIP12 was specifically required for reduction of UbG76V-GFP expression. We then examined whether the TRIP12 HECT domain affected UbG76V-GFP at the level of...
protein stability. As shown in Fig. 2C, UbG76V-GFP was destabilized by the wild-type TRIP12 HECT domain but not by the catalytically inactive mutant form. Taken together, these results suggest that the TRIP12 HECT domain recognizes the UFD substrate and that the N-terminal domain is largely dispensable for chain assembly. UBB+1 is a Substrate for TRIP12—UBB+1 is a mutant ubiquitin derived from a dinucleotide deletion in the mRNA of the ubiquitin B gene (11). UBB+1 accumulates in the brains of patients with neurodegenerative disorders, including Alzheimer disease. Although high level expression of UBB+1 inhibits the ubiquitin-proteasome system, UBB+1 at low expression levels is efficiently degraded by the proteasome (12). UBB+1 bears an N-terminal uncleavable ubiquitin moiety linked to a C-terminal extension, and the ubiquitination pattern of UBB+1 is similar to that of UFD substrates. Thus, UBB+1 has been considered a UFD substrate despite a lack of information on its E3 ligase (13). To examine whether TRIP12 is the ubiquitin ligase for UBB+1, we transfected TRIP12 knockdown cells with the expression construct for FLAG-UBB+1 and analyzed the stability of FLAG-UBB+1 after cycloheximide blockade of protein synthesis. Stability of the UBB+1 protein was significantly increased in TRIP12 knockdown cells compared with control knockdown cells (Fig. 3A). In contrast, TRIP12 knockdown did not affect the stability of UBB+1K29R/K48R, a mutant form of UBB+1 on which ubiquitination was virtually blocked. These data indicate that UBB+1 is indeed a UFD substrate ubiquitinated by TRIP12.

To test whether the TRIP12 HECT domain alone could affect stability of UBB+1, we transfected TRIP12 knockdown cells with the expression constructs for the TRIP12 HECT domain and FLAG-UBB+1. As shown in Fig. 3B, FLAG-UBB+1 was destabilized by the TRIP12 HECT domain. These results demonstrate that the HECT domain of TRIP12 recognizes and ubiquitinates UBB+1.

Knockdown of TRIP12 Reduces UBB+1-induced Cell Death in Human Neuroblastoma Cells—Previous studies have shown that high level expression of UBB+1 impairs the proteasome and causes apoptosis in neuroblastoma cells (14). Inhibition of the proteasome has been found to be dependent on ubiquitination of UBB+1 on Lys39 and Lys48 (13). To investigate the involvement of TRIP12 in UBB+1-induced cell death in human neuroblastoma SH-SY5Y cells, we analyzed the extent of cell viability and apoptosis by UBB+1 overexpression following TRIP12 knockdown. The reduction in TRIP12 expression by siRNA was confirmed by reverse transcription-PCR analysis (Fig. 4A). As shown in Fig. 4B, expression of UBB+1 reduced the viability of scrambled siRNA-treated cells to 50% compared with control transfection with empty vector. However, the UBB+1 mutant lacking both Lys39 and Lys48 did not significantly decrease cell viability, suggesting that ubiquitination of UBB+1 is required for cytotoxicity. In contrast to control knockdown cells, UBB+1 expression caused only a minor reduction (10%) in the viability of TRIP12 knockdown cells. UBB+1-induced apoptosis was also assessed by Hoechst staining. The number of apoptotic cells following UBB expression was dramatically reduced in TRIP12 knockdown cells compared with control knockdown cells (Fig. 4C). These data suggest that TRIP12 functions in ubiquitination of UBB+1, which is a prerequisite for UBB+1-induced apoptosis in neuroblastoma cells.

TRIP12 Is an E3 Ligase for UbGG—It is well established that an uncleavable ubiquitin moiety at the N terminus destabilizes the fusion protein. However, it is unclear if the nonconjugatable form of ubiquitin serves as a degradation signal independently of a C-terminal extension fused to the uncleavable ubiquitin. UbGG, a mutant ubiquitin lacking the two C-terminal glycine residues, cannot be conjugated to other proteins but can be ubiquitinated to generate an unanchored ubiquitin chain in the cell (15). Several E2 and E3 enzymes are capable of synthesizing unanchored polyubiquitin from free ubiquitin in vitro (5, 16, 17). However, the enzymes responsible for producing UbGG-terminated polyubiquitin chains in the cell have not been identified. To investigate whether UbGG could serve as a substrate for TRIP12, we analyzed the protein stability of UbGG following TRIP12 knockdown. As shown in Fig. 5A, knockdown of TRIP12 greatly stabilized FLAG-UbGG, suggesting that TRIP12 was the major ubiquitin ligase for FLAG-UbGG. We then examined whether the TRIP12 HECT domain alone could destabilize FLAG-UbGG by transfecting TRIP12
knockdown cells with the expression constructs for the TRIP12 HECT domain and FLAG-UbΔGG. Expression of FLAG-UbΔGG was lowered by the TRIP12 HECT domain but not by the catalytically inactive mutant form (Fig. 5B). The reduction of FLAG-UbΔGG by the TRIP12 HECT domain was due to the decreased protein stability of FLAG-UbΔGG (Fig. 5C). Taken together, these results indicate that the TRIP12 HECT domain recognizes the nonconjugatable ubiquitin moiety of UFD substrates and that the C-terminal extension fused to the uncleavable ubiquitin is not required for degradation of the fusion protein.

In Vitro Ubiquitination of UFD Substrates by TRIP12—To analyze TRIP12-catalyzed ubiquitination of UFD substrates directly, we set up an in vitro ubiquitination assay with recombinant proteins, which were prepared as described under “Experimental Procedures.” Fig. 6A shows staining patterns of the SDS-PAGE-resolved purified components used in ubiquitination of UbG76V-GFP. The E1 enzyme Uba1, the E2 enzyme UbcH5a, and the E3 enzyme TRIP12 were incubated with Ub G76V-GFP in the presence of ATP and ubiquitin. Ubiquitinated Ub G76V-GFP was then monitored by Western blotting with anti-GFP antibody. As shown in Fig. 6B, ubiquitination of Ub G76V-GFP required all three enzymes, confirming that TRIP12 is the human E3 ubiquitin ligase of the UFD pathway. However, the major products of the ubiquitination reaction contained only one or two ubiquitin moieties. In the yeast UFD pathway, the E3 enzyme UFD4 is capable of ligating only a few ubiquitin molecules to UbΔGG. This result supports the idea that the TRIP12 HECT domain recognizes the nonconjugatable ubiquitin moiety of UFD substrates and that the C-terminal extension fused to the uncleavable ubiquitin is not required for degradation of the fusion protein.
the substrate, and polyubiquitination of the substrate additionally requires the E4 enzyme UFD2 (7). We therefore investigated whether E4 activity similarly affects ubiquitination initiated by TRIP12. Although human UFD2a could not ligate ubiquitin to the substrate in the absence of TRIP12, it directed synthesis of long polyubiquitin chains together with E1, E2, and TRIP12 (Fig. 6C). TRIP12-catalyzed ubiquitination appeared to be stimulated by human UFD2a, since the level of unreacted UbG76V-GFP was dramatically decreased by increasing amounts of human UFD2a.

We next asked whether the TRIP12 HECT domain could ligate ubiquitin to the UFD substrate. As shown in Fig. 6D, UbG76V-GFP was modified with one or two ubiquitin molecules by the TRIP12 HECT domain in the presence of E1 and E2 and polyubiquitinated by the addition of human UFD2a to the reaction. In contrast, the E6AP HECT domain or the TRIP12 HECT domain with a cysteine to serine mutation at the conserved cysteine did not modify UbG76V-GFP (Fig. 6D, lanes 9–12). These results demonstrate that the HECT domain of TRIP12 can recognize and ubiquitinate UbG76V-GFP and that polyubiquitination of the UFD substrate requires E4 activity. To test whether UbΔGG can be ubiquitinated in vitro by the TRIP12 HECT domain, GST-UbΔGG was used as a substrate for the ubiquitination reaction. As shown in Fig. 6E, GST-UbΔGG was ubiquitinated by the TRIP12 HECT domain, and polyubiquitinated products were formed by the addition of human UFD2a to the reaction. These results confirm that the degradation signal is the nonconjugatable ubiquitin moiety of the UFD substrate, which is recognized by the HECT domain of the human UFD E3 ligase, TRIP12.

In Vivo Cross-linking of the TRIP12 HECT Domain to UFD Substrates—The direct interaction between TRIP12 and UFD substrates was tested in HeLa cells by co-immunoprecipitation methods. However, we did not observe coprecipitation of TRIP12 with the substrate, probably because the enzyme-substrate interaction was transient. To detect TRIP12 binding to the substrate in cells, we performed chemical cross-linking using DSP, a thiol-cleavable and membrane-permeable cross-linker.
HeLa cells were transfected with the expression constructs for UbG76V-GFP and FLAG-tagged HECT domain of TRIP12 or E6AP. Transfected cells were incubated with DSP in phosphate-buffered saline. The cross-linked enzyme-substrate complex was purified by immunoprecipitation with FLAG antibody-conjugated agarose and subsequently analyzed by SDS-PAGE and Western blot analysis. As shown in Fig. 7A, treatment with DSP yielded a major cross-linked product with an apparent molecular mass of 85 kDa from cells transfected with both UbG76V-GFP and TRIP12 HECT (lanes 6 and 12) but not from cells transfected with UbG76V-GFP (lanes 4 and 10) or TRIP12 HECT (lanes 5 and 11) alone. The cross-linked product was not observed without DSP treatment (lane 3) or after cleavage of the cross-linker with β-mercaptoethanol (lanes 9 and 15). In contrast to the TRIP12 HECT domain, the E6AP HECT domain did not produce cross-linked species with UbG76V-GFP (Fig. 7B), suggesting that the TRIP12 HECT domain specifically interacts with the UFD substrate.

To investigate whether the ubiquitin moiety is involved in cross-linking with the TRIP12 HECT domain, we performed cross-linking experiments with cells transfected with HA-UbΔGG and FLAG-tagged HECT domain of TRIP12 or E6AP. The TRIP12 HECT domain, but not the E6AP HECT, was cross-linked to HA-UbΔGG (Fig. 7C). Taken together, these data suggest that the ubiquitin moiety of UFD substrates is directly cross-linked to the TRIP12 HECT domain.

Substrate binding to HECT E3s involves a complementary interaction between binding motifs in the cognate substrate and the N-terminal region of the E3. For instance, the WW domain in the N-terminal region of Nedd4 interacts with PPXY motifs in substrates (18, 19). In this study, we have provided an exception to the requirement of the N-terminal portion of E3 for substrate recognition. Several lines of evidence support the conclusion that the HECT domain of TRIP12 noncovalently binds the ubiquitin moiety of UFD substrates. We found that the TRIP12 HECT domain alone can catalyze in vitro ubiquitination of UFD substrates, such as UbG76V-GFP, UBB1, and UbΔGG (Fig. 6) (data not shown). Complementation of TRIP12 knockdown cells with the TRIP12 HECT domain mostly restores efficient degradation of these UFD substrates (Figs. 2, 3, and 5), showing that the TRIP12 HECT domain can act as the E3 enzyme for UFD substrates in human cells. Moreover, we have shown that the ubiquitin moiety of UFD substrates is specifically cross-linked to the TRIP12 HECT domain (Fig. 7).

The N-terminal region of TRIP12 contains armadillo repeats and a WWE domain, both of which are presumed to mediate protein-protein interactions (20, 21). It has been recently...
Ubiquitination of UFD Substrates by the TRIP12 HECT Domain

It has been reported that the armadillo repeats of yeast UFD4 recognize the N-terminal ubiquitin of UFD substrates and are essential for ubiquitination (22). Although we cannot exclude the possibility that the armadillo repeats of TRIP12 are involved in ubiquitination of UFD substrates, our data clearly show that the TRIP12 HECT domain plays the major role in ubiquitin conjugation to UFD substrates, including recognition of those substrates. Additionally, while the armadillo repeats of yeast UFD4 are found to distinguish ubiquitin fusion proteins from free ubiquitin (22), the HECT domain of TRIP12 binds UbΔGG as a substrate (Figs. 5–7), indicating that the degradation signal recognized by TRIP12 is the nonconjugatable ubiquitin itself, not the ubiquitin fusion with a C-terminal tail. It has been shown that the N-terminal region of yeast UFD4 interacts with RPT4 and RPT6 subunits of the proteasome and that a deletion mutant UFD4 lacking the proteasome interacting region can still support ubiquitination of substrates but not their degradation by the proteasome (23). However, our data indicate that UFD substrates are ubiquitinated and destabilized by human TRIP12 HECT domain alone. Furthermore, we have not been able to detect clear interaction of TRIP12 with proteasomal ATPase subunits (data not shown). Thus, human TRIP12 and yeast UFD4 appear to employ different mechanisms for ubiquitination and degradation of their UFD substrates.

The UFD pathway is conserved from yeast to mammals (8). Therefore, it seems likely that the pathway would play an important role in eukaryotic cells. Since the pathway was first defined using artificial substrates, considerable work on the UFD pathway has been done with artificial substrates. Remarkably, UBB+/– is the one and only physiological substrate of the UFD pathway known to date. We have found that TRIP12 is the E3 for UBB+/–. Although N-terminally tagged UBB+ was used for the ubiquitination and degradation experiments shown in Fig. 3, we have observed similar results with C-terminally tagged UBB+, excluding the trivial possibility that the N-terminal FLAG tag gets cleaved off by TRIP12, mimicking degradation of UBB+ (1). In addition, we have shown that knockdown of TRIP12 reduces UBB+1-induced cell death in neuroblastoma cells (Fig. 4). Hence, one biological function of TRIP12 appears to remove the mutant ubiquitin, which could be made toxic to cells by TRIP12 itself. Although TRIP12 acts as the E3 for the UFD pathway, it is unlikely that its function is restricted to that pathway alone. Preliminary data suggest that TRIP12 ubiquitinates cellular proteins. Although the N-terminal region of TRIP12 plays a minor role, if any, in ubiquitination of UFD substrates, we expect that interaction motifs in the N-terminal region recognize the degradation signals in physiological substrates other than UFD substrates. Studies are in progress to test these hypothesized interactions.

Several E2 and E3 enzymes can catalyze the assembly of unanchored polyubiquitin chains in vitro. In addition to the activated ubiquitin molecule linked to the active site cysteine, these enzymes noncovalently bind a second ubiquitin molecule at a separate binding site. These enzymes might ubiquitinate UFD substrates, at least in vitro, provided that the C-terminal region of the substrate does not sterically hinder the interaction with the noncovalent ubiquitin binding site of the enzyme. E2 enzymes E2-2SK and the Ubc13-UEV heterodimer catalyze the synthesis of Lys+3 and Lys+6-linked polyubiquitin chains in vitro, respectively (16, 17). In the Ubc13-UEV heterodimer, the UEV protein binds an acceptor ubiquitin noncovalently, Lys+6 of which then attacks a donor ubiquitin covalently linked to Ubc13 through a thioester bond (24, 25). Recent studies using an in vitro ubiquitination system have shown that ubiquitin chain synthesis by Ubc13-UEV is greatly enhanced by the E3 TRAF6 (26). Thus, it is likely that the synthesis of free polyubiquitin chains by Ubc13-UEV in vivo is normally held in check unless stimulated by cognate E3s, to avoid unnecessary wasting of energy in the cell.

KIAA10 is a recently characterized HECT E3 that directs in vitro synthesis of unanchored polyubiquitin chains linked through Lys+9 or Lys+18 (27). KIAA10 possesses a noncovalent ubiquitin-binding site in its C-terminal domain composed of the HECT domain plus a 60-residue N-terminal extension. Because the KIAA10 C-terminal domain noncovalently binds ubiquitin as an acceptor for ubiquitin transfer, it can build up ubiquitin chains as free entities (5). Although KIAA10 catalyzes ubiquitination of UbΔGG, it is unknown whether KIAA10 can recognize and ubiquitinate other UFD substrates. Even if KIAA10 (or any other E3 with a noncovalent ubiquitin binding site) ubiquitimates UFD substrates in vitro, its contribution to the UFD pathway in the cell is likely to be minor compared with that of TRIP12, since suppression of TRIP12 expression greatly reduced degradation of the UFD substrates.

In conclusion, we demonstrated that the TRIP12 HECT domain can recognize UFD substrates and catalyze their ubiquitination. We also showed that the degradation signal recognized by TRIP12 is the nonconjugatable ubiquitin itself. These results provide new insights into the mechanism of the mammalian UFD pathway and the functional nonequivalence of different HECT domains.

REFERENCES

1. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
2. Pickart, C. (2001) Annu. Rev. Biochem. 70, 503–533
3. Hochstrasser, M. (1996) Annu. Rev. Genet. 30, 405–439
4. Scheffner, M., Nuber, U., and Hulbregtse, J. M. (1995) Nature 373, 81–83
5. Wang, M., and Pickart, C. M. (2005) EMBO J. 24, 4324–4333
6. Johnson, E. S., Ma, P. C., Ota, I. M., and Varshavsky, A. (1995) J. Biol. Chem. 270, 17442–17456
7. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999) Cell 96, 635–644
8. Dantuma, N. P., Lindsten, K., Glas, R., Jeléne, M., and Masucci, M. G. (2000) Nat. Biotechnol. 18, 538–543
9. Min, K. W., Hwang, J. W., Lee, J. S., Park, Y., Tamura, T. A., and Yoon, J. B. (2003) J. Biol. Chem. 278, 15905–15910
10. Boussif, O., Lezoualc’h, F., Zanta, M. A., Mergny, M. D., Scherman, D., Demeneix, B., and Behr, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7297–7301
11. van Leeuwen, F. W., de Kleijn, D. P., van der Hurk, H. H., Neubauer, A., Sonnemans, M. A., Sluijs, J. A., Koycu, S., Ramdijeral, R. D., Salehi, A., Martens, G. J., Grosveld, F. G., Peter, J., Burbach, H., and Hol, E. M. (1998) Science 279, 242–247
12. van Tijn, P., de Vrij, F. M., Schuurman, K. G., Dantuma, N. P., Fischer, van den Hurk, H. H., v. Leeuwen, F. W., v. Kleijn, D. P., van der Hurk, H. H., Neubauer, A., Sonnemans, M. A., Sluijs, J. A., Koycu, S., Ramdijeral, R. D., Salehi, A., Martens, G. J., Grosveld, F. G., Peter, J., Burbach, H., and Hol, E. M. (1998) J Biol. Chem. 273, 22797–22802
3 Y. Park, S. K. Yoon, and J.-B. Yoon, unpublished data.
Ubiquitination of UFD Substrates by the TRIP12 HECT Domain

D. F., van Leeuwen, F. W., and Hol, E. M. (2007) J. Cell Biol. 120, 1615–1623

13. Lindsten, K., de Vrij, F. M., Verhoef, L. G., Fischer, D. F., van Leeuwen, F. W., Hol, E. M., Masucci, M. G., and Dantuma, N. P. (2002) J. Cell Biol. 157, 417–427

14. de Vrij, F. M., Sluijs, J. A., Gregori, L., Fischer, D. F., Hermens, W. T., Goldgaber, D., Verhaagen, J., van Leeuwen, F. W., and Hol, E. M. (2001) FASEB J. 15, 2680–2688

15. Arnason, T., and Ellison, M. J. (1994) Mol. Cell. Biol. 14, 7876–7883

16. Chen, Z., and Pickart, C. M. (1990) J. Biol. Chem. 265, 21835–21842

17. Hofmann, R. M., and Pickart, C. M. (2001) J. Biol. Chem. 276, 27936–27943

18. Kanelis, V., Rotin, D., and Forman-Kay, J. D. (2001) Nat. Struct. Biol. 8, 407–412

19. Ingham, R. J., Gish, G., and Pawson, T. (2004) Oncogene 23, 1972–1984

20. Coates, J. C. (2003) Trends Cell Biol. 13, 463–471

21. Aravind, L. (2003) Trends Biochem. Sci. 26, 273–275

22. Ju, D., Wang, X., Xu, H., and Xie, Y. (2007) FEBS Lett. 581, 265–270

23. Xie, Y., and Varshavsky, A. (2002) Nat. Cell Biol. 4, 1003–1007

24. VanDemark, A. P., Hofmann, R. M., Tsui, C., Pickart, C. M., and Wolberger, C. Cell (2001) 105, 711–720

25. Moraes, T. F., Edwards, R. A., McKenna, S., Pastushok, L., Xiao, W., Glover, J. N. M., and Ellison, M. J. (2001) Nat. Struct. Biol. 8, 669–673

26. Petroski, M. D., Zhou, X., Dong, G., Daniel-Issakani, S., Payan, D. G., and Huang, J. (2007) J. Biol. Chem. 282, 27936–279945

27. You, J., and Pickart, C. M. (2001) J. Biol. Chem. 276, 19871–19878