Identification of a Novel 29-Linked Polyubiquitin Binding Protein, Ufd3, Using Polyubiquitin Chain Analogues[†,‡]

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ABSTRACT: Lysine 48-linked polyubiquitin chains are the best understood form of polyubiquitin and are necessary for the function of the ubiquitin−proteasome system. However, other forms of polyubiquitin (e.g., K29- and K63-linked chains) are also present in vivo. Less is known about the functional roles of these linkages or the proteins specifically interacting with these forms of polyubiquitin. Use of native polyubiquitin chains to identify binding proteins is complicated by the difficulties of synthesis and stability. Here, we report the synthesis of a nonhydrolyzable analogue of 29-linked polyubiquitin chains on an affinity support and its use in identifying proteins that bind 29-linked polyubiquitin chains. The 29-linked Ub_4 resin was stable and tightly bound recombinant human Isopeptidase T (USP5), a deubiquitinating enzyme known to bind the 29-linked polyubiquitin chains. Two high affinity interactors of the 29-linked polyubiquitin analogues were identified from *Saccharomyces cerevisiae* lysates. They were identified as Ubp14, the yeast ortholog of Isopeptidase T, and Ufd3, a member of the ubiquitin-fusion degradation pathway with unknown function. Purified recombinant Ufd3 bound to the resin as well, confirming that Ufd3 is a novel binding partner of polyubiquitin. These results demonstrate the efficacy of using polyubiquitin analogue affinity supports to identify novel binding partners of specifically linked polyubiquitin chains. Identification of these proteins will lead to a greater understanding of the physiological relevance of different polyubiquitin linkages.

Ubiquitin is an essential 76 amino acid protein found in all eukaryotes and highly conserved from yeast to humans (*1*). One of ubiquitin's functions is to target various cellular proteins to the 26S proteasome for degradation (*2*, *3*). This is done by covalently attaching the C terminus of ubiquitin to the ϵ-amino group of a lysine on a target protein or another ubiquitin. This conjugation is performed by the E1/E2/E3 enzymatic cascade and results in the formation of polyubiquitin chains on the targeted protein (*4*, *5*). E1 is charged with ubiquitin in an ATP-dependent step that results in a high-energy thiolester linkage between the E1 and the ubiquitin molecule. The ubiquitin is then transferred to an E2, retaining the thiolester linkage. The charged E2 will usually interact with an E3 ubiquitin ligase, and the ubiquitin is then linked to a target protein or to another ubiquitin to form a polyubiquitin chain. Numerous E2s and E3s present in the cell provide specificity as to when and how a target protein is ubiquitinated (*6*, *7*).

A polyubiquitin chain of four residues is sufficient to target proteins to the proteasome for degradation (*8*). Polyubiquitin chains containing an isopeptide linkage between a C-terminal glycine of one ubiquitin and the K48 residue of another are essential to the degradation of proteins by the ubiquitin−proteasome pathway. Overexpressing a K48R mutant ubiquitin (a polyubiquitin chain terminator) in *Saccharomyces cerevisiae* as the sole source of ubiquitin is lethal to the yeast (*9*, *10*). However, there are six other lysine residues in ubiquitin, and polyubiquitin chains can be formed using all of these residues in vitro (*11*). Recently, chains linked through all seven lysine residues have been detected in vivo with K29- and K63-linked chains, the most characterized forms of alternatively linked polyubiquitin (*12*). Despite the detection of non-K48-linked polyubiquitin chains, they have not been wellcharacterized in relation to their structure, function, or binding partners.

K63-linked polyubiquitin chains appear to have functions distinct from targeting proteins to the proteasome for proteolysis. K63-linked chains are believed to act as signaling molecules in diverse cellular pathways including endocytosis, stress response, and DNA repair (*13*−*15*). The E2 Mms2/Ubc13 and E3 TRAF6 were the first E2/E3 pair identified that specifically synthesized K63-linked chains (*16*, *17*). In addition to TRAF6 (which undergoes autoubiquitination), L28, TRAF2, and PCNA are modified by the attachment of 63-linked polyubiquitin chains in vivo (*18*−*20*). However, Mms2/Ubc13 and TRAF6 are not responsible for 63-linked polyubiquitin conjugation to L28, indicating that there are other E2/E3 pairs capable of synthesizing 63-linked polyubiquitin chains. Nothing is known about the proteins that interact with these chains once they have been conjugated to a substrate.

Even less is known about the role of K29-linked polyubiquitin chains in vivo. Conjugating enzymes that synthesize 29-linked polyubiquitin chains have been purified from

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[‡] The described DNA sequence of the *Xenopus laevis* homologue of Isopeptidase T was submitted to GenBank under the accession number AY376839.

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human cell lines. The E2 responsible is UbcH5, and the E3 is KIAA10 (*21*), although KIAA10 is also capable of synthesizing K48-linked chains. It is not clear if there is an E2/E3 pair exclusively devoted to synthesizing K29-linked chains. However, K29-linkages are still formed in a yeast deletion strain lacking Hul5, the yeast homologue of KIAA10, indicating other proteins are capable of making K29-linked polyubiquitin. Functionally, K29-linked chains have only been shown to have a role in the ubiquitin-fusion degradation (UFD) pathway (*22*). This pathway was initially discovered in *S. cerevisiae* during a genetic screen for defects in proteasomal degradation of proteins with N-terminally fused ubiquitins. Some of these substrates required at least one ubiquitin linked to the K29 residue of the N-terminally fused ubiquitin for degradation to proceed. Despite the degradation requirement for this linkage, its role is unknown. Although there is genetic evidence for K29-linked chains interacting with other proteins, direct biochemical evidence is limited (*23*). Because of the limited number of known binding partners of K29 chains, it has been difficult to elucidate biological functions for these chains.

To begin to understand these alternatively linked polyubiquitin chains, we synthesized nonhydrolyzable ubiquitin dimer analogues that mimicked various chain linkages (e.g., 48, 63, 29, and 11 linked) (*24*). They were found to be useful as stable and specific inhibitors of various enzymes of the ubiquitin−proteasome system, including conjugating enzymes and deubiquitinating enzymes with polyubiquitin binding sites. The inhibitory effect of the dimer analogues was dependent on the chain linkage incorporated into the dimer. Although our work with dimer analogues demonstrated them to be good general inhibitors of ubiquitin conjugation and deconjugation, it did not identify the specific interactions with other proteins that caused these inhibitory effects.

To identify proteins that bind specifically to polyubiquitin chains, we have modified our dimer synthesis protocol to synthesize longer nonhydrolyzable polyubiquitin chain analogues on an affinity support. We have used tetrameric chains, with the minimum length required for recognition by the proteasome and the length preferred by other polyubiquitin chain-binding proteins (*8*, *25*). We report here the synthesis of a 29-linked nonhydrolyzable ubiquitin tetramer analogue resin and its use to identify the two major binding partners of the 29-linked polyubiquitin chains in *S. cerevisiae* whole-cell lysates, Ubp14 (Isopeptidase T) and Ufd3 (a novel polyubiquitin binding protein). Thus, affinity supports containing nonhydrolyzable analogues of polyubiquitin chains can serve as useful tools for identifying novel interactions between proteins and non-K48-linked polyubiquitin chains.

MATERIALS AND METHODS

General. The MHY501 yeast strain (gift of M. Hochstrasser) was used to make yeast lysates, and the ACY192 strain (gift of A. Corbett) was used for expression of His-FLAG-Ufd3. Anti-Ufd3 antibody and the His-FLAG-Ufd3 expression vector were a kind gift from M. Ghislain. Standard molecular biology techniques were used for all cloning procedures.

Double Point Mutant Ubiquitins. Plasmids encoding K29C and G76C ubiquitin were made as previously described (*24*). A vector expressing the double mutant (K29C/G76C ubiquitin) was constructed using vector/insert ligation of *Afl*II/*Nde*I digested fragments from the appropriate single mutant ubiquitin plasmids. The appropriate DNA fragments were purified by gel extraction (Qiagen). The K29C *Afl*II/*Nde*I insert (in 10-fold molar excess) and G76C vector backbone were joined in a 20-μL ligation reaction. The reaction contained T4 DNA ligase buffer and 5×-concentrated T4 DNA ligase (New England Biolabs) plus DNA in a 20-min reaction at room temperature. Concentrated ligase was used because of the difficulty in ligating *Afl*II sites. Ligated DNA was transformed into DH5α cells and recovered on LB-ampicillin plates. Colonies were picked and amplified, and the plasmid DNA was then purified and sequenced.

29-Linked Ub_4 Resin Synthesis. K29C, G76C, and the double mutant (K29C/G76C) ubiquitins were purified as previously described (*24*). To begin analogue resin synthesis, 10 mg of K29C ubiquitin was added to 10 mL of 0.1 M sodium phosphate at pH 6.8 and 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to block the cysteine residue resulting in K29C-TNB ubiquitin. After 10 min at room temperature, the reaction mixture was dialyzed against pH 7.4 phosphate-buffered saline overnight at 4 °C with three buffer changes. 6-aminohexanoic acid *N*-hydroxysuccinimide ester Sepharose 4B (1.5 g) was then incubated with the dialyzed K29C-TNB ubiquitin at room temperature, and the binding of ubiquitin to resin was analyzed by following the loss of ubiquitin in the unbound fraction by high-performance liquid chromatography (HPLC). Binding was complete in 1 h. Unreacted sites on the resin were blocked by incubation in 1 M ethanolamine for 1 h at room temperature. After 1 h, β-mercaptoethanol was added to a final concentration of 50 mM, and the mixture was incubated for an additional 30 min to remove the TNB blocking group.

The derivatized resin was washed with 10 column volumes of 20 mM sodium acetate at pH 5.0. To extend the chain, the resin was resuspended in 10 mL of 70 mM borate at pH 8.3 and 1,3-dichloroacetone (DCA) (Sigma) was added in 10−20-fold molar excess of ubiquitin bound to the resin. The resin was incubated for 10 min at 4 °C with gentle agitation and then washed with 10 column volumes of 70 mM borate at pH 8.3 to remove excess DCA. K29C/G76C ubiquitin in 5 mL of 70 mM borate at pH 8.3 was added to the resin in 1.5-fold molar excess over the immobilized ubiquitin. The mixture was incubated for 2 h at 4 °C with gentle agitation. β-mercaptoethanol was added to a final concentration of 50 mM, and the mixture was incubated for an additional 30 min at 4 °C. To prepare for the next round of ubiquitin addition, the resin was washed with 3 column volumes of 20 mM sodium acetate, 1 M NaCl at pH 5.0, and 10 column volumes of 20 mM sodium acetate at pH 5.0. In the final conjugation step, G76C ubiquitin was used instead of K29C/G76C ubiquitin. All conjugation steps were monitored by HPLC to determine the yield.

Yeast Lysates. Yeast strain MHY501 was used to make whole-cell lysates. Yeast extract, peptone, dextrose (YPD) media (5 mL) was inoculated with colonies picked from YPD plates and grown at 30 °C to an absorbance at 600 nm (OD_{600}) of 0.6. This starter culture was used to inoculate 1.5 L of fresh YPD. After inoculation, the culture was grown to an OD_{600} of 1.5. Cells were pelleted, washed with distilled water, and repelleted. Cell pellets were frozen in liquid

nitrogen and then ground to a powder by mortar and pestle cooled in liquid nitrogen. The powder was resuspended in lysis buffer containing 0.1% (v/v) Triton X-100, 50 mM Tris-HCl at pH 7.2, 150 mM NaCl, 2 mM dithiothreitol (DTT), and 100 μM phenylmethylsulfonylfluoride (PMSF). A protease inhibitor cocktail consisting of pepstatin A, leupeptin, aprotinin, and chymostatin was also added to a final concentration of 3 μg/μL each. Crude lysate was centrifuged at 4000 rpm for 5 min, and the supernatant was collected. The pellet was refrozen in liquid nitrogen and then reground. Two cycles of grinding achieved at least 50−60% lysis of cells determined by light microscopy. Crude supernatants from each grinding cycle were pooled and then centrifuged in a SS-34 rotor at 10 000 rpm for 30 min at 4 °C to remove unlysed cells and cellular debris. Supernatant from the spin was then used in subsequent experiments. Protein concentration of the lysate was determined by Bradford reagent and was generally 15−20 mg/mL.

Affinity Chromatography. Lysate (10 mL) was incubated with 500 μL of 29-linked Ub_4 resin (generally containing about 1−1.5 mg of tetramer) overnight at 4 °C with gentle shaking. After incubation, the slurry was poured into a column and washed with 12 column volumes of lysis buffer and 18 column volumes of lysis buffer without Triton. The column was sequentially eluted by high-salt buffer (lysis buffer and 1 M NaCl) and then stripped with 8 M urea and 1 mM HCl buffer. Fractions of 1 mL were collected, and 500 μL of each fraction was precipitated with trichloroacetic acid (TCA) at a final concentration of 10%, resuspended in 20 μL of 50 mM Tris-HCl at pH 8.0, and then analyzed on a 10% SDS−PAGE gel. Protein was detected by staining with Sypro Ruby Red (Molecular Probes) protein dye. Bands to be analyzed were excised from the gel and sent to the Emory Microchemical facility for mass spectroscopy (MS) analysis.

Trypsin Digest and Matrix-Assisted Laser Desorption Ionization (MALDI) Identification. Protein bands were excised from SDS−PAGE gels and cut into small cubes. Gel cubes were subjected to three 15-min washes with 10 mM NH_4HCO_3 in 50% acetonitrile (v/v). Gel cubes were then dried under vacuum for 30 min with no heat and rehydrated with 5 μL of 10 mM NH_4HCO_3 plus 0.5 μL of 0.5 μg/μL sequencing-grade modified trypsin (Promega). An additional 10 mM NH_4HCO_3 was slowly added until gel cubes had swelled to their original size and then were incubated overnight at 37 °C. The reaction was stopped by addition of 1.5 μL of 10% trifluoroacetic acid (TFA). Supernatant was removed, and the peptides were extracted from the gel cubes by three 25-min incubations with 10 mM NH_4HCO_3 and 60% acetonitrile. The supernatant and extracts were pooled and concentrated under vacuum to a volume of approximately 10 μL.

For MALDI, a C18 Zip Tip (Millipore) was washed with a 50% HPLC-grade water and 50% acetonitrile (v/v) solution and then equilibrated with a 0.1% TFA solution. An aliquot of the tryptic digest was acidified with 10% TFA and then added to the Zip Tip. The Zip Tip was then washed with 0.1% TFA and eluted with 0.6 μL of 70% acetonitrile, 0.1% TFA, and 1 μL of α-cyano-4-hydroxycinnamic acid matrix. The eluates were spotted directly onto a Bruker MALDI target and then analyzed by a Bruker Reflex III MALDI-TOF-MS in reflector mode. Synthetic peptides were used for internal calibration. Monoisotopic peptide peaks were analyzed with the NCBI nonredundant database using Profound (http://129.85.19.192/profound_bin/WebProFound.exe).

LC-MS-MS Analysis of Analogue Binding Proteins. Samples were prepared for analysis by TCA precipitation of the urea eluate from the 29-linked Ub_4 resin and washing the protein pellet with acetone. After air-drying, the sample was sent to the Yale Cancer Center Mass Spectrometry Resource and then dissolved in 20 μL of 8 M urea and 0.4 M NH_4HCO_3 at pH 7.5−8.5. DTT (5 μL of 45 mM) was then added, and the sample was incubated at 37 °C for 20 min. Next, 5 μL of 0.1 M iodoacetamide was added, and the sample was incubated at room temperature for 20 min. After incubation, 45 μL of distilled water plus 5 μL of 0.1 mg/mL trypsin were added, and the sample was incubated at 37 °C for 24 h. The sample was then adsorbed onto a Zip Tip for LC-MS-MS analysis.

Human Isopeptidase T Purification. Human Isopeptidase T (IsoT) cDNA was amplified by the polymerase chain reaction from a human fetal brain cDNA library and rescued by the TA Cloning System as previously described (*26*). The rescued IsoT cDNA was cloned into the pRSET vector by use of vector/insert ligation using *Nde*I and *Hind*III digested pRSET vector and IsoT cDNA. pRSIsoT was then expressed in BL21(DE3) cells. Single colonies were used to inoculate starter cultures in LB−ampicillin (100 μg/mL) media that were grown to mid-log phase (OD_{600} = 0.6−0.8) at 37 °C. A total of 50 mL of starter culture was used to inoculate each 1.5 L of LB−ampicillin media. A total of 12 L of culture were grown to mid-log phase at 37 °C. Cells were centrifuged, and the pellets were resuspended in 12 L of fresh 42 °C LB−ampicillin media. Cells were heat shocked with shaking at 42 °C for 30 min. After heat shock, cultures were induced with 10 μM isopropyl-β-D-thiogalactopyranoside (Denville Scientific) and incubated for 40−48 h at 15 °C. After induction, cells were pelleted and frozen at −80 °C.

Cell pellets were thawed and resuspended in 150 mL of lysis buffer [50 mM Tris-HCl at pH 8.0 and 25 mM ethylenediaminetetraacetic acid (EDTA)] per liter of cells. β-mercaptoethanol was added to a final concentration of 10 mM, and a protease inhibitor cocktail was added [100 μM benzamidine, 0.5 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 μg/mL chymostatin, 2 μg/mL antipain, and 2 μg/mL aprotinin (all final concentrations)]. Lysozyme was also added to a final concentration of 100 μg/mL. After a 20 min incubation at room temperature, the cell lysate was sonicated in a 2020 Hert Systems Ultrasonics sonicator (six cycles of 30 s bursts at power level 6 followed by 1 min of rest on ice). The lysate was centrifuged at 12 000 rpm in a Sorvall GSA rotor for 20 min, and supernatants were collected. The supernatant was then directly loaded onto a 100 mL Fast Flow Q column (Amersham) equilibrated with buffer containing 20 mM Tris-HCl at pH 7.6, 10 mM β-mercaptoethanol, and 5 mM EDTA. After loading, the column was washed with 400 mL of column buffer and then eluted with a 500-mL linear gradient from 0 to 350 mM NaCl. Isopeptidase T activity in fractions was monitored by HPLC as previously described using ubiquitin ethyl ester as the substrate (*27*).

Fractions with high activity were pooled and loaded onto 5 mL of ubiquitin−Sepharose containing 12 mg/mL ubiq-

uitin. The monoubiquitin (monoUb) resin was equilibrated with 10 column volumes of 0.5 M KCl, 50 mM Tris-HCl at pH 7.6, and 2 mM DTT buffer. After loading, the resin was washed with 5 column volumes of the above buffer and then eluted with 25 mM ethanolamine and 10 mM DTT at pH 9.4. Fractions were neutralized to pH 7.0 with 3 M sodium acetate, and those containing high enzymatic activity were pooled and concentrated using a Millipore YM-30 filter. Concentrated Isopeptidase T was dialyzed into 50 mM Tris-HCl at pH 7.5 and 10 mM β-mercaptoethanol. After gel filtration on Sephacryl S-200 resin equilibrated with dialysis buffer, the protein was concentrated, aliquoted, and frozen at −80 °C.

Nonhydrolyzable Ubiquitin Dimer Synthesis. Dimers were made as previously described (*24*).

Recombinant Isopeptidase T Binding Experiments. Purified Isopeptidase T (0.1 mg) was diluted to 500 μL in yeast lysis buffer (50 mM Tris-HCl at pH 7.2, 150 mM NaCl, and 2 mM DTT) and incubated with 100 μL of 29-linked Ub_4 resin for 90 min at 4 °C with gentle shaking. An identical experiment using ethanolamine blocked N-hydroxysuccinimide-activated (NHS-activated) Sepharose (i.e., no conjugated ubiquitin) was used as a negative control. After incubation, reactions were centrifuged, and the supernatants were removed and saved for SDS−PAGE analysis. Resin-bound Isopeptidase T was batch-eluted by boiling in 500 μL of SDS-sample buffer. Bound and unbound fractions were analyzed by SDS−PAGE and Coomassie staining to estimate the amounts of Isopeptidase T present. In competition experiments, nonhydrolyzable dimers were added to the resin before addition of Isopeptidase T.

Ufd3 Purification. The yeast strain ACY192 was transformed with the pFLAG-Ufd3 plasmid expressing His-FLAG-Ufd3 (*28*). A total of 5 mL starter cultures were grown to saturation in trp-synthetic media and 2% glucose. Cells were recovered by centrifugation, and then each pellet was resuspended in 1 L of trp-synthetic media with 2% raffinose. When the culture reached an OD_{600} of 1.0, galactose was added to a final concentration of 2% and the culture was induced for 6 h. Cell pellets were lysed by the liquid nitrogen method described, except for the lysis buffer used, which was (500 mM NaCl, 50 mM Na_2HPO_4, 10 mM β-mercaptoethanol, 100 μM PMSF, 0.1% (v/v) Triton X-100, and 10 mM imidazole at pH 8.0). Lysate was then incubated with 3 mL of Ni−NTA Agarose (Qiagen) equilibrated with lysis buffer for 1 h at 4 °C with gentle stirring. The slurry was poured into a column and washed with lysis buffer containing 20 mM imidazole at pH 8.0. Protein was eluted with lysis buffer containing 250 mM imidazole at pH 8.0. Direct binding and competition experiments were performed as described for Isopeptidase T, except that binding was allowed to proceed for 3−15 h.

Anti-Ufd3 Western Blots. Yeast lysates were prepared by the liquid nitrogen lysis method. A total of 200 μL of 29-linked Ub_4 resin and 500 μL of the appropriate yeast lysate were incubated overnight to test native and recombinant Ufd3 binding. Experiments containing only recombinant His-FLAG-Ufd3 used 10 μg of protein. When it was added to a wild-type lysate, only 1 μg was used. Samples of each experiment were run on 10% SDS−PAGE gels and transferred to nitrocellulose in 20 mM Tris-HCl, 150 mM glycine, and 20% methanol at pH 8.0 for 1 h at 100 V. Anti-Ufd3 was prepared as described previously (*28*). The primary antibody was detected with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (Amersham) followed by enhanced chemiluminescence detection (Amersham).

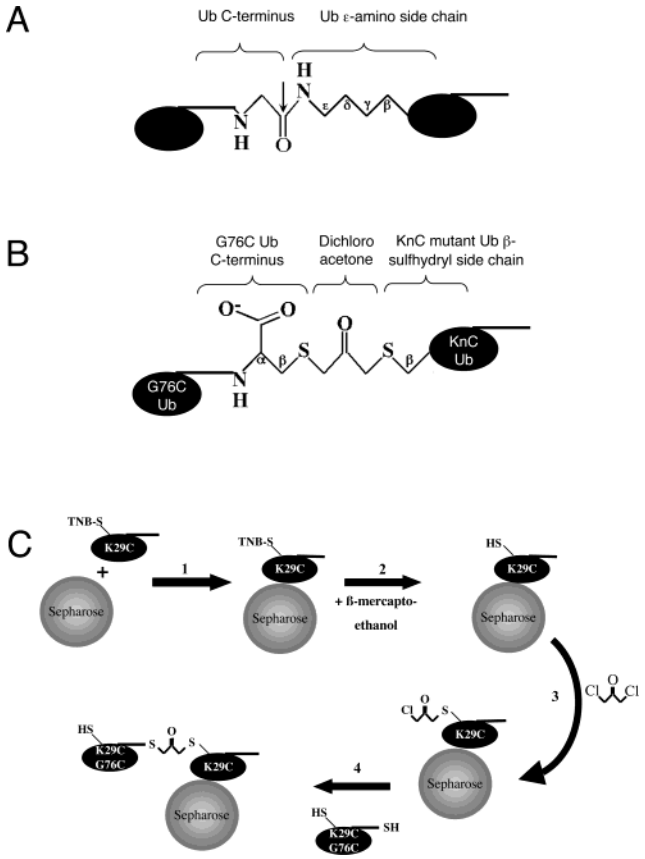

FIGURE 1: Structure of polyubiquitin and analogues. (A) Chemical structure of the native isopeptide bond in a polyubiquitin chain. Cleavage of this bond by DUBs takes place at the arrow. (B) Chemical structure of the nonhydrolyzable linkage between ubiquitins in our polyubiquitin analogues. There is one extra carbon in this linkage compared to the native isopeptide bond. (C). Synthesis of the 29-linked polyubiquitin analogue resin. (1) K29C ubiquitin with the cysteine residue blocked with TNB is coupled to NHS-activated Sepharose. The cysteine is blocked to prevent it from coupling to the Sepharose, which would prevent polyubiquitin chain extension. (2) β-mercaptoethanol (50 mM) is added at the end of the blocking with ethanolamine to remove the TNB from the cysteine residue, making it available for chain extension. (3) The resin is washed and then reacted with 10−20-fold molar excess of DCA (relative to ubiquitin bound to resin) for 10 min to derivatize the C29 residue. (4) After washing, the derivatized resin is incubated with 1.5-fold molar excess of K29C/G76C ubiquitin to form the dimer. Steps 3 and 4 are then repeated until the tetramer-length polyubiquitin is synthesized onto the resin.

RESULTS

Synthesis of 29-Linked Nonhydrolyzable Polyubiquitin Analogue Resin. To facilitate synthesis of 29-linked polyubiquitin chain analogues, we mutated K29 and/or the C-terminal glycine residue of ubiquitin to cysteine to allow cross-linking with DCA. Parts A and B of Figure 1 compare normal isopeptide-linked and DCA cross-linked ubiquitins. The appropriate ubiquitin mutants were purified and used to synthesize tetramer analogues onto activated Sepharose resin as shown in Figure 1C. Each ubiquitin conjugation step was monitored by HPLC to determine the amount of ubiquitin bound to the resin or growing polyubiquitin chain.

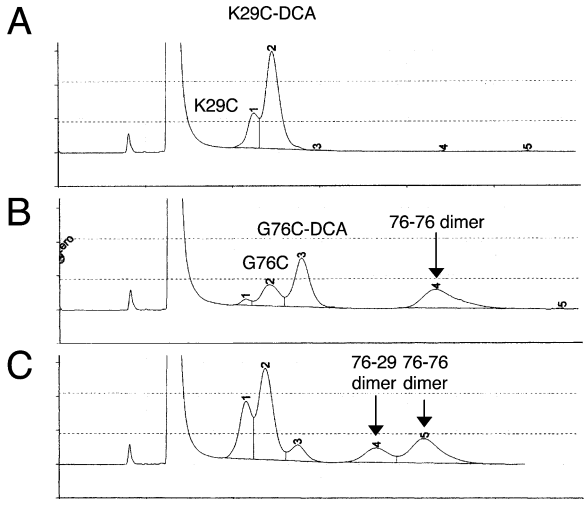

FIGURE 2: Specificity of the DCA cross-linking reaction. (A and B) Ubiquitin mutants (0.2 mg of either K29C or G76C ubiquitin) were diluted to 100 μL in 70 mM sodium borate at pH 8.3. An equimolar amount of DCA was added, and the reaction was allowed to proceed for 1 h on ice. A 5 μL aliquot was then analyzed by HPLC. Panel A shows the K29C reaction where no ubiquitin dimer forms. Panel B shows the G76C reaction where the 76−76 dimer is readily formed. (C) Dimerization reaction between K29C-DCA and an equimolar amount of G76C. G76C was reacted with K29C-DCA for 2 h in 70 mM sodium borate at pH 8.3 on ice. Because there is unreacted DCA present, it can react with G76C and allow for the formation of the 76−76-linked dimer as well as the 76−29-linked dimer. This would not occur in resin synthesis because excess DCA is removed before conjugation. Note: The large peak eluting before ubiquitin in the HPLC chromatograms results from the *N*,*N*-dimethylformamide that the dichloroacetone is dissolved in.

For the first ubiquitin addition, the DTNB blocking step prevented the cysteine residue of K29C ubiquitin from conjugating to the resin. Thus, K29C-TNB ubiquitin conjugated to the resin through lysine residues, most likely K6 (*29*). Substitution achieved in this step was 2.5 mg of ubiquitin/ 1 mL of resin.

To initiate the second cycle of addition, the blocking group was removed with β-mercaptoethanol and the resin-bound ubiquitin was subsequently reacted with DCA. Addition of K29C/G76C ubiquitin resulted in 83% of the resin-bound monoUb being converted to ubiquitin dimer. Because the K29C residue is much less reactive than the G76C residue and is the only DCA-activated residue available for conjugation (see parts A−C of Figure 2), only the desired C76−C29 linkage is produced. Two more cycles of DCA activation and ubiquitin conjugation resulted in the formation of the resin-bound tetramer (29-linked Ub_4 resin) linked through residue 29 with the proximal ubiquitin of the chain attached to the resin. Average yields for each individual step were as follows: Conjugation of K29C to Sepharose resin was defined as 100%. An average yield of approximately 80% coupling was observed for the second and third ubiquitins added and >95% for conjugation of the fourth ubiquitin. Percent yield is based on the amount of resin-bound ubiquitin available for cross-linking at each step in the conjugation process. The total yield of the tetramer in relation to the initial ubiquitin monomer added to Sepharose was calculated to be 62.4%.

Characterization of 29-Linked Ub_4 Resin. After synthesis of the 29-linked Ub_4 resin, it was rigorously tested to ensure

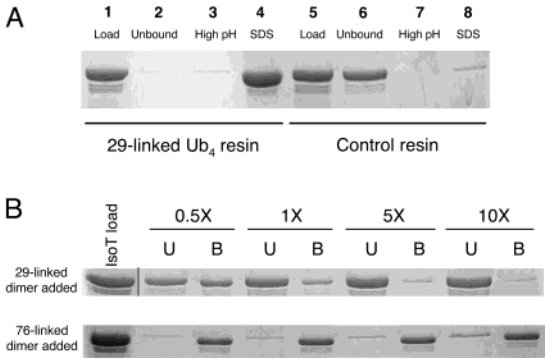

FIGURE 3: Linkage specificity of Isopeptidase T binding to 29-linked Ub_4 resin. (A) Isopeptidase T is specifically bound to the 29-linked Ub_4 resin. Isopeptidase T was incubated with aliquots of 29-linked Ub_4 or control resin (containing no ubiquitin) for 90 min. The unbound fractions were removed (lanes 2 and 6), and the resins were washed, then eluted with 25 mM ethanolamine, 10 mM DTT buffer at pH 9.4 (lanes 3 and 7), and then boiled in 200 μL of 1× SDS-sample buffer (lanes 4 and 8). Equivalent fractions were loaded onto a 10% SDS−PAGE gel and Coomassie Blue stained. (B) The Isopeptidase T binding experiment was repeated in the presence of varying amounts of either the 76−29-linked or 76−76-linked nonhydrolyzable ubiquitin dimer. A $^1/_2$−10-fold molar ratio (in relation to the amount of Isopeptidase T added) of each dimer was used as indicated. After incubation, the resin was washed and then eluted by boiling in 500 μL of 1× SDS-sample buffer. U = unbound fraction and B = bound fraction eluted with SDS-sample buffer. Gels are stained with Coomassie Blue.

that it was synthesized correctly and capable of specifically binding proteins that can interact with 29-linked polyubiquitin chains. Base hydrolysis of 29-linked Ub_4 resin followed by SDS−PAGE analysis clearly showed that polyubiquitin was present on the resin (data not shown). However, the DCA linkage between ubiquitins and the amide bond between ubiquitin and Sepharose were hydrolyzed at similar rates, so accurate quantitation of the relative amounts of different length polyubiquitins on the resin was impossible.

The 29-linked Ub_4 resin was then tested to see if it could specifically bind a protein that is known to bind 29-linked polyubiquitin chains, human USP5 (referred to herein as Isopeptidase T) Isopeptidase T is a deubiquitinating enzyme conserved from yeast to humans. Its main function is to disassemble free polyubiquitin chains into monomeric ubiquitin, and it is known to cleave K29-linked polyubiquitin chains (data not shown). To test binding specificity, 100 μL of 29-linked Ub_4 resin equilibrated in 150 mM NaCl, 50 mM Tris-HCl at pH 7.2, and 2 mM DTT buffer was combined with 0.1 mg of purified Isopeptidase T diluted to 500 μL in equilibration buffer. As a negative control, an equal amount of Isopeptidase T was incubated with Sepharose that had been prepared in an identical manner to the 29-linked Ub_4 resin, except that no ubiquitin was coupled to it.

Figure 3A demonstrates that all Isopeptidase T added binds to the 29-linked Ub_4 resin, while no Isopeptidase T binds to the control resin (compare lanes 4 and 8). This illustrates that Isopeptidase T is binding to polyubiquitin and not interacting nonspecifically with the Sepharose bead or the ethanolamine used to block the Sepharose. Isopeptidase T does not completely bind to a monoUb resin under similar conditions (data not shown). This preference for binding polyubiquitin over monoUb suggests that Isopeptidase T is interacting with the tetramer analogue and not the residual monoUb present on the analogue resin. Purified Isopeptidase

T binds so avidly to the 29-linked Ub_4 resin that urea or SDS is required to elute it. This tight binding correlates well with our previous data demonstrating that 29-linked nonhydrolyzable ubiquitin−dimer binds with a low nanomolar binding affinity to Isopeptidase T, which is 1000-fold greater than the affinity of Isopeptidase T for monoUb (*24*). These results demonstrate the 29-linked Ub_4 resin is specifically recognized and bound by proteins capable of interacting with 29-linked polyubiquitin chains.

To confirm that linkage specificity is important in Isopeptidase T binding to polyubiquitin, we repeated the Isopeptidase T binding experiment in the presence of different nonhydrolyzable ubiquitin dimers as competitors. The 29-linked dimer and a nonphysiological C-terminally fused dimer (76−76 linked) were tested. Previous results showed that they were the most and least effective inhibitors, respectively, of Isopeptidase T enzymatic activity, with a 100-fold difference in their apparent K_i values (*24*). As Figure 3B demonstrates, the 29-linked dimer effectively inhibited binding of Isopeptidase T to the 29-linked Ub_4 resin. Addition of equimolar amounts of 29-linked dimer prevented Isopeptidase T binding to the 29-linked Ub_4 resin. The 76−76-linked dimer was an ineffective inhibitor, even at 10-fold molar excess. This demonstrates that linkage specificity is a significant contributor to polyubiquitin recognition and binding by Isopeptidase T.

Identification of Yeast Proteins Binding to 29-Linked Ub_4 Resin. To identify 29-linked polyubiquitin chain binding proteins from crude extracts, we performed binding experiments using *S. cerevisiae* lysates. Budding yeast were chosen because of the ease in obtaining large quantities of soluble protein and complete knowledge of its genome, which facilitates identification of potential chain binding proteins in the lysate. A large amount of protein was used (300−400 mg of protein/ 1 mL of resin) to ensure identification of as many potential 29-linked polyubiquitin chain binding proteins as possible. Yeast lysate was incubated with 500 μL of 29-linked Ub_4, monoUb, or control resin overnight at 4 °C and then washed extensively. Figure 4A shows that very little protein was bound to any of the resins (compare lanes 1 and 2). After washing, each column was eluted with high-salt buffer (1 M NaCl) followed by 8 M urea. Figure 4B demonstrates that there was very little nonspecific binding to the Sepharose resin. Comparison of parts C and D of Figure 4shows that the two bands (at approximately 95 and 70 kDa) are present in multiple elution fractions from the 29-linked Ub_4 resin and are highly enriched compared to equivalent fractions in the monoUb or control resin experiments (compare Figure 4D to parts B and C of Figure 4). Thus, the 95 and 70 kDa bands were specifically bound to the 29-linked polyubiquitin analogues.

The 95 and 70 kDa bands were excised from the gel and subjected to trypsin digestion and MALDI-MS analysis. Table 1 shows the MALDI-MS data for the 95 kDa band, unambiguously identifying the protein as Ubp14, the yeast homologue of Isopeptidase T. Of the 26 tryptic peptides analyzed by MALDI-MS, 17 were matched to Ubp14, and these peptides covered 27% of the Ubp14 sequence. These data confirm Ubp14, a known K29-linked polyubiquitin binding protein, is a major yeast protein bound to the 29-linked Ub_4 resin and serves as an internal positive control.

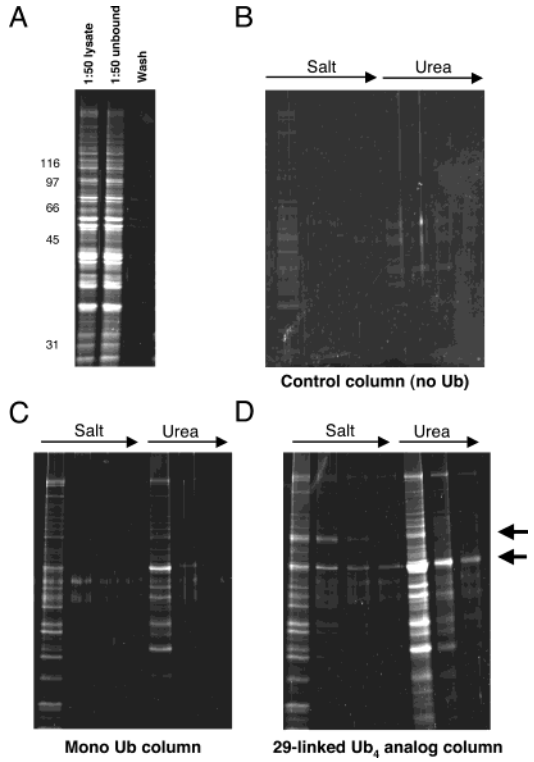

FIGURE 4: Identifying the binding partners of 29-linked polyubiquitin chain analogues in yeast whole-cell extract. All experiments used approximately 150−200 mg of soluble protein. Sequential 1 mL elution fractions were collected. Fifty percent of each eluted fraction was TCA precipitated, resuspended, and loaded onto a 10% SDS−PAGE gel. Gels were then stained with Sypro Ruby Red protein dye. (A) Representative gel of the protein added, unbound, and washed fractions for all yeast lysate experiments. (B) Binding experiment using control resin (no ubiquitin). (C) Binding experiment using monoUb resin. (D) Binding experiment using 29-linked Ub_4 resin. Bands highlighted by arrows indicated candidate polyubiquitin binding proteins.

Table 2 shows that the 70 kDa protein band was identified by MALDI-MS analysis as Ufd3 (also known as Doa1 or Zzz4). Out of 33 tryptic peptides analyzed, 12 were matched to Ufd3, covering 22% of the Ufd3 sequence. Ufd3 was originally identified as a protein involved in the UFD pathway. Interestingly, some of the ubiquitin-fusion protein substrates required addition of a ubiquitin in a K29 linkage to the fused ubiquitin in order for the substrate's degradation to take place (*22*). Thus, both Ubp14 and Ufd3 bound tightly to the 29-linked Ub_4 resin, consistent with the genetic and physical evidence that they are involved in K29-linked polyubiquitin metabolism.

To identify additional 29-linked Ub_4 resin binding proteins from yeast, we analyzed the yeast proteins eluted with urea by electrospray LC-MS-MS (Table 3). Ufd3 was abundant in the urea fraction. A total of 13 peptides matching 21.5% of the Ufd3 sequence were identified from this sample. Two human ubiquitin peptides were found as well. The ubiquitin probably comes from small amounts of tetramer leaching off during urea elution. Other proteins identified included highly abundant yeast proteins such as alcohol dehydrogenase, pyruvate kinase, and elongation factor 1-α. Comparison of the lysate lane in Figure 4A and first urea lane in Figure 4D shows that the pattern of proteins is very similar. The similarity indicates that most of the bands seen in the urea

Table 1: MALDI Identification of Ubp14 Bound to 29-Linked Ub_4 Resin from Yeast Lysates[a]

measured mass (Da)	expected mass	missed cuts	error (%)	residues	peptide sequence
827.302	827.330	0	−0.003	469−474	FMMEDR
870.482	870.518	0	−0.004	78−84	HVPLHIR
931.402	931.451	0	−0.005	142−149	FNGENVPR
1065.442	1065.513	0	−0.007	774−781	WVLYNDEK
1106.532	1106.583	0	−0.005	253−262	SNNNHPLAIK
1195.502	1195.566	0	−0.005	794−802	NGYIYFYTR
1325.672	1325.741	1	−0.005	692−703	KALILNNGDVNR
1331.572	1331.639	0	−0.005	512−522	AYFEGQTIEFK
1363.792	1363.818	0	−0.002	540−551	SLPQTLILNPIR
1413.702	1413.746	0	−0.003	562−574	TSNELSLPGLIDR
1518.652	1518.663	0	−0.001	421−433	CIGQNHQEFSSNR
1570.762	1570.800	0	−0.002	752−765	GNSVHSGHYVVFIR
1822.892	1822.884	0	0.000	462−468	FFSSSSSGIPNPNDLVR
1846.832	1846.870	0	−0.002	237−252	EQIGIDGHSHALDHYR
2030.862	2031.016	0	−0.008	310−325	TLVQLQVEQNENWQFR
2041.002	2040.982	0	0.001	85−102	VTEYACDTIHSNYLTIAK
3008.152	3008.358	0	−0.004	485−509	YSYEPTEAIQIPLEENDEPQDMLER

[a] All masses are monoisotopic. Peptides covered 27% of the sequence. *Z* score was 2.36. Data were obtained using Version 4.10.5 of ProFound found at http://129.85.19.192/profound_bin/WebProFound.exe.

Table 2: MALDI Identification of Ufd3 Bound to 29-Linked Ub_4 Resin from Yeast Lysates[a]

measured mass (Da)	expected mass	missed cuts	error (%)	residues	peptide sequence
791.382	791.392	0	−0.001	700−706	SYGNVPR
933.472	933.492	0	−0.002	534−541	TPAYDIVR
967.372	967.449	0	−0.008	316−323	TIEFDESK
1243.612	1243.630	0	−0.001	173−183	TFSGIHNDIVVR
1358.712	1358.719	0	0.000	324−335	LSPYEILQSPGR
1645.832	1645.744	0	0.005	215−227	TYEGHESFVYCIK
1716.972	1716.940	0	0.002	670−685	LTVAYGNLATVEPTLR
1772.782	1772.846	0	0.001	598−613	SIFETIDTEFSQASAK
1902.892	1902.885	0	0.000	345−361	SPQGTIEAHQFSNSSWK
2019.872	2019.851	0	0.001	654−669	YGPLEEYQECEEAAYR
2090.062	2090.042	0	0.001	402−420	LPINVSDNPYTAADNFLAR
2101.012	2101.022	0	0.000	126−144	EGSLVYNLQAHNASVWDAK
2113.022	2113.025	0	0.000	384−401	TYDYVFDVDIEDGKPPLK

[a] All masses are monisotopic. Peptides cover 22% of the sequence. *Z* score was 2.34. Data were obtained using Version 4.10.5 of ProFound found at http://129.85.19.192/profound_bin/WebProFound.exe.

Table 3: Mass Spectrometry Data of Urea Elution Fraction from 29-Linked Ub_4 Resin[a]

protein identified	Lynx score	peptides matched	% coverage	MW (Da)	description
DOA1	1477.72	10	19.3	79 487	Ufd3
ADH1	***	6	***	36 826	alcohol dehydrogenase
HS72	707.58	5	10.81	69 320	heat-shock protein Ssa2
HS71	671.56	5	10.76	69 508	heat-shock protein Ssa1
EF1A	989.43	4	14.85	50 014	elongation factor 1-α
MPG1	513.12	3	10.8	39 547	mannose-1-phosphate guanyl transferase
KPY1	186.31	3	7.6	54 526	pyruvate kinase
UBIQ	248.21	2	32.89	8546	ubiquitin
RSP5	***	1	***	91 760	RSP5, E3 ubiquitin ligase

[a] Data obtained at Yale Cancer Center Mass Spectrometry Resource, Yale University, New Haven, CT. Matching peptides were identified from MS/MS spectra manually and automatically using the Mascot algorithm and Proteinlynx searching algorithm. The Lynx score derives from the Proteinlynx algorithm. The triple asterisks indicate that because of manual identification of the peptides, a Lynx score was not obtained.

fraction are not enriched upon binding to the 29-linked Ub_4 resin and are thus likely the result of nonspecific binding.

In addition to the abundant proteins discussed above, three other proteins related to the ubiquitin system were identified, Ssa1, Ssa2, and Rsp5. It is unclear as to whether the Ssa proteins are bound specifically or nonspecifically. There is some evidence for this class of proteins being involved in ubiquitin-mediated proteolysis and other cellular pathways where ubiquitin is involved (*30*, *31*). However, Ssa proteins are notorious for being nonspecific interactors in other affinity purification experiments, so more investigation will be necessary (*32*). Another interesting result of the LC-MS-MS analysis was the identification of a peptide from Rsp5, a yeast ubiquitin ligase (*33*). However, because only a single peptide was observed, this identification is tenuous.

A similar binding experiment was also performed with *Xenopus* egg lysates (data not shown). The only protein specifically bound to the 29-linked Ub_4 resin and identified was the *Xenopus* homologue of Isopeptidase T. Because the *Xenopus* Isopeptidase T sequence was not present in the databases, MALDI analysis could not directly identify the protein as Isopeptidase T. However, because the molecular weight of the adsorbed protein was reasonable, we obtained a full-length *Xenopus* cDNA clone (IMAGE clone 3200767),

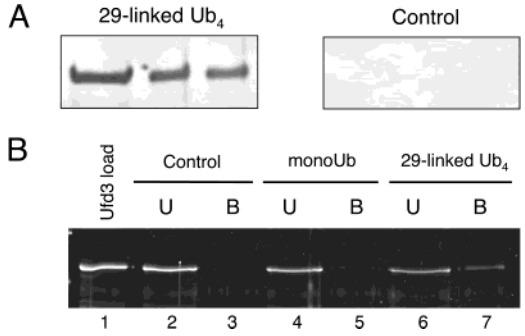

FIGURE 5: Ufd3 is a polyubiquitin protein binding protein. (A) Sequential salt elution fractions from the 29-linked Ub_4 or control resin were run on SDS−PAGE and immunoblotted with an anti-Ufd3 antibody. (B) Purified His-FLAG-Ufd3, diluted to 500 μL in lysate buffer, was incubated with the control, monoUb, or 29-linked Ub_4 resins. After incubation, the unbound fraction was removed, and then the resin was washed and then batch-eluted with 500 μL of 8M urea. Samples were analyzed on a 10% SDS-PAGE gel stained with Sypro Ruby Red. Ufd3 is clearly present in the urea elution fraction from the 29-linked Ub_4 resin but only minimally in the control or monoUb eluates.

whose expressed sequence tags were homologous to human Isopeptidase T, and sequenced it. The sequence was deposited in GenBank (accession number AY376839) and used to determine potential tryptic digest sites. Numerous peptide masses were identical to those found in the tryptic digest of our sample, confirming the identification of *Xenopus* Isopeptidase T.

Characterization of the Ufd3 Interaction with Polyubiquitin. We performed several experiments to confirm and characterize the interaction of Ufd3 with polyubiquitin. To confirm the MALDI-MS identification, anti-Ufd3 antibody was used to immunoblot protein eluted from the 29-linked Ub_4 resin and corresponding control resin fractions. Figure 5A shows Ufd3 is present in the 29-linked Ub_4 resin elution fractions but not in the control resin fractions, thus confirming the MALDI-MS identification.

Direct binding experiments were then performed using purified Ufd3 to assess if Ufd3 was binding directly to polyubiquitin. To obtain recombinant Ufd3, we expressed His-FLAG-tagged Ufd3 from a yeast expression vector in *S. cerevisiae* and purified it using Ni−NTA agarose resin (Qiagen). Purified His-FLAG-Ufd3 was then incubated with aliquots of 29-linked Ub_4, monoUb, or control resin. Figure 5B demonstrates that purified His-FLAG-Ufd3 is a direct polyubiquitin binding protein, although much of the His-FLAG-Ufd3 was present in the unbound fraction. To test whether unbound His-FLAG-Ufd3 was nonfunctional or capable of binding polyubiquitin, unbound fractions from the initial binding experiment were incubated with fresh 29-linked Ub_4 resin (data not shown). Results of this second-pass experiment were identical to the first experiment; i.e., a fraction of the applied protein was again bound. Thus, Ufd3 binds directly to the polyubiquitin analogue. This result suggests that the purified His-FLAG-Ufd3 was functional but had a relatively low affinity for the 29-linked Ub_4 resin.

To determine why purified recombinant Ufd3 appeared to bind more weakly than endogenous Ufd3 (in the lysate experiments), we performed a number of binding experiments. To test if there was a factor in the lysate that aided the Ufd3 binding to the 29-linked Ub_4 resin, we made a lysate from a ΔUfd3 deletion strain (Invitrogen) and added purified

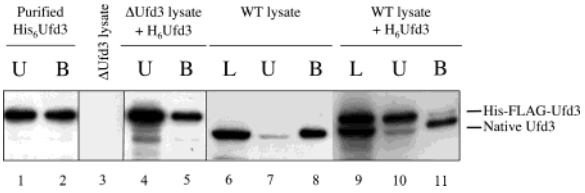

FIGURE 6: N-terminally tagged Ufd3 is partially functional. Direct binding experiments were performed with purified HIS-FLAG-Ufd3, endogenously expressed Ufd3, or both forms. All fractions were appropriately diluted for equal loading and analyzed by SDS−PAGE and immunoblotting with anti-UFD3 antibody. Ufd3 (5 ng) was present in each lane. L = lysate fraction before incubation with 29-linked Ub_4 resin. U = unbound fraction after incubation. B = bound fraction eluted off 29-linked Ub_4 resin. Lanes 1−5 show that the recombinant Ufd3 is able to bind to the analogue resin. Lanes 6−11 show that endogenous Ufd3 is much more efficiently bound than the recombinant Ufd3.

recombinant Ufd3 to the lysate before incubating with the 29-linked Ub_4 resin. Figure 6 shows that the proportion of bound His-FLAG-Ufd3 bound to the 29-linked Ub_4 resin was not increased in the presence of lysate (compare lanes 2 and 5). Lanes 6−8 show an experiment where only wild-type lysate was incubated with the 29-linked Ub_4 resin. Almost all of the endogenous Ufd3 is bound to the resin, a significantly different result from the recombinant His-FLAG-Ufd3 experiments.

To directly compare recombinant His-FLAG-Ufd3 binding versus endogenous Ufd3 binding, wild-type lysate was supplemented with an amount of His-FLAG-Ufd3 equal to the amount of endogenous Ufd3 present. Virtually all of the native Ufd3 was bound to the 29-linked Ub_4 resin, while only a small portion of the His-FLAG-Ufd3 was bound. This is a clear indication that the recombinant protein is not fully functional and could be the explanation for the apparently weaker binding seen with His-FLAG-Ufd3 (see Discussion). Also, we noted that the bound endogenous Ufd3 migrates slightly slower compared to the unbound band (lanes 7 and 8). However, this apparent difference is an artifact because of the presence of Triton X-100 in the lysate and unbound fractions but not in the elution fractions (data not shown).

DISCUSSION

In recent years, it has become clear that polyubiquitin has additional roles beyond targeting proteins for proteasomal degradation. Polyubiquitin has been shown to act as a signaling molecule in endocytic, DNA repair, and transcriptional regulation pathways. Polyubiquitin chains linked through residues other than K48 were demonstrated to have involvement in some of these pathways, notably K63-linked chains. If differently linked chains have specificity for different subsets of proteins, then it would be expected that different polyubiquitin chains have different structures. Recent work indicating that ubiquitin dimers with different linkages appear to have different defined quaternary structures bolsters this idea (*34*, *35*). To better characterize the role of K29-linked polyubiquitin, we have identified interacting proteins through the synthesis of nonhydrolyzable polyubiquitin analogues onto affinity supports.

Synthesis and Characterization of an Affinity Support for Polyubiquitin Binding Proteins. We report here the synthesis of stable nonhydrolyzable polyubiquitin chain analogues conjugated to an affinity support. Cross-linking ubiquitins with dichloroacetone, a bifunctional thiol reagent, yielded

the desired form of polyubiquitin analogue. Cross-linking was controlled via the introduction of cysteine at appropriate residues in the ubiquitin sequence because no cysteines are in the wild-type sequence. DCA cross-linked chain analogues are not susceptible to cleavage by deubiquitinating enzymes (DUBs), although the chain analogues are bound by DUBs, inhibiting their enzymatic activity (*24*). The linkage introduced by cross-linking is one carbon longer than the physiological linkage. However, structural studies of polyubiquitin chains have shown the isopeptide linkage region to be disordered or in an extended conformation, so an extra carbon may not have a significant effect (*36*). Spatial orientation of the ubiquitins in a chain is faithfully mimicked in the analogues and may be the most important factor for preserving the individual geometry of the differently linked polyubiquitin chains. The stability of these chain analogues allows us to analyze crude extracts for proteins that bind specifically linked polyubiquitin chains.

The first polyubiquitin chain analogue that we have synthesized on an affinity support is a ubiquitin−tetramer that is linked through residue 29. We analyzed this form of polyubiquitin because its functional role has been poorly characterized and 29-linked chain analogues were the easiest to synthesize by our method of solid-phase synthesis. Because of the limited reactivity of the C29 residues due to steric effects, a nonphysiological 29−29 ubiquitin−ubiquitin linkage is not formed in our synthesis (see parts A−C of Figure 2). Solid-phase synthesis gives good yields with little evidence of heterogeneity in the length of the polyubiquitin chain.

The 29-linked Ub_4 resin synthesized was thoroughly tested for its ability to act as a good mimic of the physiological form of K29-linked polyubiquitin. The 29-linked Ub_4 resin was found to contain the right form of chain linkage and interact with recombinant Isopeptidase T (Figure 3A), a known K29-linked polyubiquitin binding protein, very strongly. Conditions that elute Isopeptidase T from monoUb resin did not elute Isopeptidase T from the tetramer resin, suggesting that underivatized monoUb present on the resin is not present in an orientation that allows interaction with ubiquitin binding proteins.

To further test that the 29-linked Ub_4 resin faithfully mimicked the physiological form, competition binding experiments with Isopeptidase T and multiple nonhydrolyzable ubiquitin dimers were performed (see Figure 3B). We demonstrated that the 29-linked Ub_4 resin does have linkage specificity based on the results of these experiments. The 29-linked dimer effectively competed for Isopeptidase T binding, while the nonphysiological 76−76 dimer was ineffective at competing for Isopeptidase T binding. These data imply that our synthesized polyubiquitin chain analogues faithfully mimic the spatial orientation of ubiquitins in native chains. The results, although not quantitative, also demonstrate Isopeptidase T's unusual ability to recognize specific linkages in polyubiquitin chains. How Isopeptidase T recognizes all of the forms of polyubiquitin found in vivo with different specificities is still unclear. Quantitative binding or kinetic studies on Isopeptidase T cleavage of native chains are currently planned. Structural studies on Isopeptidase T would also provide insight into how ubiquitin binding proteins interact with polyubiquitin chains.

Purification of Polyubiquitin Binding Proteins from Lysates. After confirming the utility and linkage specificity of our 29-linked Ub_4 resin, we analyzed crude whole-cell extracts from *S. cerevisiae*. Few abundant binding partners were found in the extract. One major binding partner of the 29-linked Ub_4 resin found in the yeast extract was Ubp14. This result was not surprising because of the abundance of Ubp14 in these cells and the high affinity that the recombinant human Isopeptidase T had for the Ub_4 resin. These results suggest that Ubp14 is a major DUB able to bind 29-linked polyubiquitin chains in yeast and confirm the usefulness of the 29-linked Ub_4 resin.

The most abundant binding partner of the 29-linked Ub_4 resin found in *S. cerevisiae* extracts was Ufd3, also known as Doa1 or Zzz4. We have also demonstrated that purified His-FLAG-Ufd3 binds polyubiquitin directly. However, the amount of purified His-FLAG-Ufd3 binding to the 29-linked Ub_4 resin is considerably less than that of endogenous Ufd3. Our analysis of endogenous Ufd3 binding in lysates shows that almost all of the Ufd3 present in the lysate binds to the resin even when recombinant Ufd3 is present in equal amounts (Figure 6). The fact that almost all of the endogenous Ufd3 in the lysate interacts with the resin in our experiments suggests that the interaction between Ufd3 and K29-linked polyubiquitin is significant. The difference in binding affinity between endogenous and recombinant Ufd3 suggests that the epitope tag or inefficient folding of the recombinant protein may interfere with the binding to polyubiquitin. Upon further analysis of native Ufd3 binding to our chain analogue, we were unable to inhibit binding to 29-linked Ub_4 resin with molar excess of various nonhydrolyzable ubiquitin dimers (data not shown). This could be because Ufd3 prefers interacting with longer forms of polyubiquitin. Because we do not yet have nonhydrolyzable tetramer analogues in solution, we have not been able to test this hypothesis.

Is There a Role for Ufd3 in the Ubiquitin Pathway? Ufd3 was originally identified during a screen for defects in ubiquitin-mediated degradation of N-terminal ubiquitin-fusion proteins. Deletion phenotypes in yeast include lower levels of cellular-free ubiquitin, defects in sporulation, and resistance to certain volatile anesthetics (*28*, *37*). Analysis of the Ufd3 sequence shows that its N-terminal half contains six WD40 repeats and that the protein is highly homologous to a mammalian protein annotated as phospholipase A2 activating protein (PLAP). Phospholipase activation by Ufd3 has not been demonstrated in *S. cerevisiae*, and its physiological function in yeast is unknown. The C-terminal half of Ufd3 contains a (Vps27/Hrs/STAM)(VHS) domain, a domain found in proteins involved in endocytosis and membrane trafficking (*38*−*40*). The VHS domain in the C-terminal half of the protein is unusual because the VHS domain is usually found at the N terminus. A FxDxF adaptin α-ear binding motif used in interacting with clathrin-coated vesicles is present at the C terminus of the protein (*41*). Curiously, Ufd3 does not have a recognizable ubiquitin-binding domain (ubiquitin-associated domain, ubiquitin-interacting motif, etc.), despite its ability to interact with polyubiquitin. Although speculative, the domain analysis and homology to PLAP suggest a role for Ufd3 in some form of vesicle or membrane trafficking where polyubiquitin is required as a signal molecule. Further characterization of Ufd3 will be necessary to test this idea.

Utility of the Analogue Columns in Identifying Linkage-Specific Polyubiquitin Chain Binding Proteins. Our results

have identified two major chain binding proteins from yeast lysates, Ubp14 and Ufd3. They are significant interactors because they bound virtually completely to the 29-linked Ub_4 resin when yeast lysates were analyzed. Are there others? A number of other abundant proteins are bound to the 29-linked Ub_4 resin and washed off with urea. However, because these proteins are not obviously enriched in the eluate, it is likely that they are nonspecifically bound. An MS/MS analysis of the complete urea-eluted fractions shows that two Ssa-family chaperones were present and that Rsp5, a ubiquitin ligase, might also be present. However, identification of these proteins as specific interactors of K29-linked polyubiquitin was tenuous and needs to be confirmed. Other proteins that bind 29-linked chains might be more difficult to identify if binding depended on interactions with a protein to which the 29-linked polyubiquitin chain is conjugated. Despite significant amounts of protein applied to the resin, low abundance chain binders could be missed because they would be present in amounts too small to be detected by gel staining, which is necessary for MALDI-MS identification. Alternatively, there just may not be many 29-linked polyubiquitin chain binders in the cell. However, the method presented here is the only method available to perform a global screen for novel binders of polyubiquitin chains linked through a specific lysine residue.

There was limited overlap between proteins interacting with our 29-linked Ub_4 resin and K48-linked polyubiquitin interactors. No proteasomal subunits, ubiquitin-specific processing proteases (other than Ubp14), Cdc48, or other known chain binding proteins were identified in these experiments. Proteasomes and Cdc48 are abundant in yeast and should have been identified if they were bound to the Ub_4 resin. The lack of interactions with these types of proteins indicates the specificity of binding to polyubiquitin chains with different chain linkages, although experimental conditions potentially play a role as well. The ability of a protein to distinguish between chains of different linkage would allow such chains to act as signaling molecules in additional pathways other than proteasomal degradation.

Recent work by other groups provides data demonstrating the importance of linkage specificity and how tools such as our polyubiquitin analogue resin could be useful in answering questions about the functional roles of non-K48-linked polyubiquitin chains. Recent crystal structures and molecular modeling of the Mms2/Ubc13 heterodimer involved in synthesizing K63-linked chains have shown that this complex exhibits molecular specificity (*42*). This specificity only allows synthesis of K63-linked chains. Modeling studies have suggested that this E2 cannot interact with ubiquitin in such a way as to form K48-linked polyubiquitin chains. Chen et al. have also demonstrated alternatively linked polyubiquitin involved in a nonproteasomal pathway (*43*). Their studies have shown that BRCA1/BARD1, an E3 ubiquitin ligase, stabilizes itself by autoubiquitination. This paradox was resolved by determining that these polyubiquitin chains are not K48-linked chains. It is not clear what linkages are in these chains although K29, K63, and most recently K6 linkages have been proposed (*44*, *45*). It will be interesting to further characterize Ufd3 and its functional relationship with K29-linked polyubiquitin to further elucidate what role this interaction has in the cell.

We have used a novel approach to identify polyubiquitin binding proteins: the synthesis of a nonhydrolyzable polyubiquitin analogue affinity resin. The identification and characterization of polyubiquitin chain-interacting proteins will help to define functional roles for K29-linked and other forms of polyubiquitin chains and the signaling pathways that they help regulate. Therefore, it will now be of interest to extend these studies to the identification of binders of K6-, K11-, K48-, and K63-linked polyubiquitin analogues.

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