Ubiquibodies, Synthetic E3 Ubiquitin Ligases Endowed with Unnatural Substrate Specificity for Targeted Protein Silencing*

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Background: Techniques that harness the power of the ubiquitin-proteasome pathway (UPP) for protein knock-out are limited to a narrow set of protein targets.

Results: Engineered “ubiquibodies” specifically and systematically removed exogenous target proteins.

Conclusion: Diverse protein targets can be redirected to the UPP using this new protein silencing method.

Significance: Ubiquibodies offer a simple, reproducible, and customizable technique for selectively and controllably depleting cellular proteins.

The ubiquitin–proteasome pathway (UPP) is the main route of protein degradation in eukaryotic cells and is a common mechanism through which numerous cellular pathways are regulated. To date, several reverse genetics techniques have been reported that harness the power of the UPP for selectively reducing the levels of otherwise stable proteins. However, each of these approaches has been narrowly developed for a single substrate and cannot be easily extended to other protein substrates of interest. To address this shortcoming, we created a generalizable protein knock-out method by engineering protein chimeras called “ubiquibodies” that combine the activity of E3 ubiquitin ligases with designer binding proteins to steer virtually any protein to the UPP for degradation. Specifically, we reprogrammed the substrate specificity of a modular human E3 ubiquitin ligase called CHIP (carboxyl terminus of Hsc70-interacting protein) by replacing its natural substrate-binding domain with a single-chain Fv (scFv) intrabody or a fibronectin type III domain monobody that target their respective antigens with high specificity and affinity. Engineered ubiquibodies reliably transferred ubiquitin to surface exposed lysines on target proteins and even catalyzed the formation of biologically relevant polyubiquitin chains. Following ectopic expression of ubiquibodies in mammalian cells, specific and systematic depletion of desired target proteins was achieved, whereas the levels of a natural substrate of CHIP were unaffected. Taken together, engineered ubiquibodies offer a simple, reproducible, and customizable method for directly removing specific cellular proteins through accelerated proteolysis.

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§ The abbreviations used are: scFv, single-chain Fv; uAb, ubiquibody; TPR, tetratricopeptide repeat; β-gal, β-galactosidase; CHIP, C terminus of Hsc70-interacting protein.

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be further compounded if the association between the DBP and its target protein is short-lived, leading to "escape" of the target and thus ineffective inhibition. Even when high-affinity binders are available, target inactivation is not guaranteed because not all DBPs are endowed with "intrinsic-neutralizing" properties. Thus, we sought to develop a protein silencing strategy that links a DBP with the natural degradation machinery of the cell, the ubiquitin proteasome pathway (UPP), such that the steady-state levels of the intended target of the DBP are systematically reduced.

The UPP is the main route of protein degradation in eukaryotic cells and is a common mechanism through which numerous cellular pathways are regulated (8). Most proteins are targeted for proteasomal degradation after being covalently modified with a polyubiquitin chain, which is attached to either internal lysine residues or the N-terminal residue on target proteins. This polyubiquitin serves as the signal for recognition and proteolytic destruction by the 26S proteasome or, in some cases, by lysosomes. The canonical cascade of ubiquitination involves three enzymes termed E1, E2, and E3. The substrate specificity of a given ubiquitin-proteolytic pathway is conferred by the E3 (9), whose intrinsic structural flexibility enables accommodation of substrates with different sizes and structures (10). This conformational flexibility along with the fact that "consensus" ubiquitination sites are not required for ubiquitin transfer (11) raises the distinct possibility that E3s may be engineered for accelerating the degradation of unnatural target proteins of interest. Indeed, several methods have been reported for steering cellular proteins to the UPP for degradation. Most of these have been based on a multimeric E3 complex called SCF, which consists of Skp1 (S-phase kinase-associated protein 1), Cul-1, F-box-containing substrate receptor, and RING proteins. The F-box domain of the substrate receptor binds to Skp1 to connect to the core SCF complex, whereas a protein-protein domain (such as the WD40 domain or leucine rich repeat) of the receptor is responsible for substrate recruitment. Protein knock-out using the SCF system has been developed by introducing established protein-protein interaction modules to the F-box-containing substrate receptor for specific recruitment of target proteins (12, 13) or using peptide-small-molecule hybrids, known as ProtaCts, that bridge the interaction between the intended target and the F-box (14). Although these studies demonstrate that SCF-type E3s can be used to eliminate specific protein targets, the approach requires the endogenous SCF core machinery to carry out ubiquitination. It is possible, therefore, that the core ligase machinery might become overwhelmed upon high level expression of engineered F-box proteins, thereby suppressing the degradation of both the intended target as well as native substrates (15). A related concern is the complexity of SCF complex formation, which requires the presence of numerous protein components in a very precise molecular ratio.

A viable alternative is the use of "stand-alone" E3s that bind their substrates and transfer ubiquitin to these substrates without the need for additional accessory factors. For example, the single-chain U-box type E3 called CHIP (carboxyl terminus of Hsc70-interacting protein) has been redesigned for ubiquitination of specific targets (10, 16, 17). However, nearly all engineered E3s described so far, including both U-box and SCF-types, rely on a naturally existing binding partner to interact with the desired target protein. As a result, engineered E3s have only been developed for a limited set of substrates and cannot be easily extended to other protein substrates of interest. Causinus et al. (13) recently addressed this issue by engineering a chimera comprised of the SCF-type E3 F-box domain and a single-domain antibody fragment (i.e., nanobody) specific for GFP, which was capable of depleting target GFP fusions. Although the engineered chimera still targeted just a single substrate, namely the GFP domain of a target fusion protein, the development of "GFP protein trap" methods in cultured cells (18) and even whole, live organisms (19) makes it possible to target a wide array of GFP-tagged proteins. However, because silencing requires the targets to be recombinantly fused to GFP, this method is not able to deplete natural targets in the absence of the GFP domain. The F-box-nanobody fusions were also unable to process GFP on its own, suggesting that engineered SCF systems might be constrained by a size limit below which silencing may not be possible.

To create a method for depletion of virtually any target protein, we engineered chimeras whereby DBPs were fused to the C-terminal U-box ligase domain of human CHIP. We chose CHIP for several reasons. First, unlike SCF-based systems, CHIP does not rely on other subunits for its functionality. Second, CHIP has a broad substrate diversity (20), suggesting that its specificity can be altered without affecting its ubiquitin transfer activity. Third, CHIP is modular in nature, and its U-box domain is known to remain active in fusions (10, 16, 17). Our idea was that a target protein recognized by the DBP domain would be specifically degraded upon ectopic expression of the engineered fusion; hence, we call these fusions ubiquibodies (uAbs) because of their potential for antibody-mimetic binding and ubiquitination of target proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Full-length human CHIP (a gift from Cam Patterson) was PCR amplified for cloning into pET28a(+) using 5′ Ncol and 3′ SalI restriction sites. DNA encoding a double tag of FLAG-His₆ was created by dimerizing primers with a 5′ SalI overhang and a 3′ HindIII overhang. Double ligation was performed to insert the CHIP PCR product and primer dimer between Ncol and HindIII sites in pET28a(+), yielding plasmid pET28a-CHIP. To create truncated CHIPΔTPR, DNA corresponding to amino acids 128–303 of human CHIP was PCR amplified with introduction of a 5′ Ncol site and a 3′ SalI site. Double ligation was performed as above to insert this product along with the primer dimer into pET28a(+), yielding pET28a-CHIPΔTPR. The genes encoding scFv13 and scFv13-R4 (a gift from Pierre Martineau) were PCR-amplified, and each was double ligated into pET28a(+) with the above primer dimer, yielding the control plasmids pET28a-scFv13 and pET28a-scFv13-R4, respectively. To create CHIPΔTPR fusions, PCR was used to introduce an EcoRI site following a short, flexible linker of GSΣG to the 5′ end of CHIPΔTPR. In parallel, each of the DBPs including scFv13, scFv13-R4, and the scFv D10 (a gift from Andreas Plückthun) was PCR-amplified with a 5′ Ncol site and 3′ EcoRI site. Double ligation was then used to insert each single-chain Fv (scFv) along with the GSΣG linker-CHIPΔTPR product between the Ncol and SalI sites.
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sites of pET28a-CHIP, yielding pET28a-scFv13-uAb, pET28a-R4-uAb, and pET28a-D10-uAb. Due to an internal EcoRI site in the FN3 monobody YS1 (a gift from Shohei Koide), overlap extension PCR was used to add the GSGSG linker and N terminus of CHIPATPR to the YS1 PCR product, until it reached a unique BstBI site within CHIPATPR. This overlap extension PCR product was then ligated between NcoI and BstBI sites of pET28a-R4-uAb, yielding pET28a-Y51-uAb. The R272A point mutation was introduced into pET28a-R4-uAb using a QuikChange site-directed mutagenesis kit (Stratagene). For expression in eukaryotic cells, the above constructs were PCR-amplified from their pET28a(+) backbones using primers that introduced a Kozak sequence at the start codon as well as a 5′ HindIII site for MBP or 5′ XhoI for β-gal and a 3′ XbaI site for both and cloned in the corresponding sites of pcDNA3. Due to an internal Sall site, the YS1 gene was directly cloned into pcDNA3 using overlap extension to add a HindIII site and Kozak sequence to the 5′ end. The unique BstBI site within CHIP TPR. This overlap extension primers that introduced a Kozak sequence at the start codon as well as a 5′ HindIII site and XbaI sites of plasmid pcDNA3. Due to an internal Sall site, the YS1 gene was directly cloned into pcDNA3 using overlap extension to add a HindIII site and Kozak sequence to the 5′ end and FLAG tag. His tag and XbaI site to the 3′ end. The target substrate proteins β-gal and maltose-binding protein (MBP) were PCR-amplified using primers that introduced a Kozak sequence at the start codon as well as 5′ HindIII site for MBP or 5′ XhoI for β-gal and a 3′ XbaI site for both and cloned in the corresponding sites of pcDNA3.

Protein Expression and Purification—All purified proteins were obtained from cultures of Escherichia coli BL21 (DE3) cells grown in 500 ml of Luria-Bertani medium. Expression was induced with 0.1 mM IPTG when the culture density (A600) reached 0.6–0.8 and proceeded at 30 °C for 6 h, after which cells were harvested by centrifugation at 4,000 × g for 20 min at 4 °C. The resulting pellets were stored at −80 °C overnight. Thawed pellets were resuspended in 15 ml buffer A (20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole, pH 7.4) and lysed with a high-pressure homogenizer (Avestin Emulsiflex C5). Lysates were cleared by centrifugation at 20,000 × g for 20 min at 4 °C and then subjected to Ni2⁺-affinity purification with an ÄKTA FPLC using a 1 ml HiTrap column (GE Healthcare). Samples were washed with 10% buffer B (20 mM sodium phosphate, 0.5 M NaCl and 50 mM imidazole, pH 7.4) before elution with 50% buffer B. Purified proteins were desalted over a 5-ml HiTrap column equilibrated with ubiquitination reaction buffer (20 mM MOPS, 100 mM KCl, 1 mM DTT, 5 mM MgCl2, pH 7.2).

In Vitro Ubiquitination Assay—Ubiquitination assays were performed as described previously (10) in the presence of 0.1 μM purified human recombinant UBE1 (Boston Biochem), 2 μM human recombinant UbcH5α/UBE2D1 (Boston Biochem), 3 μM uAb (or equivalent control protein), 3 μM E. coli β-gal (Sigma), 50 μM human recombinant ubiquitin (Boston Biochem), 4 mM ATP, and 1 mM DTT in 20 mM MOPS, 100 mM KCl, 5 mM MgCl2, pH 7.2. Reactions were carried out at 37 °C for 2 h (unless otherwise noted) and stopped by boiling in 2× Laemmli loading buffer for analysis by immunoblotting.

Mass Spectrometry Analysis—For LC-MS/MS sample preparation, ubiquitination assays were performed as described previously (21) but with 0.1 μM UBE1, 20 μM UbcH5α, 20 μM R4-uAb, 20 μM β-gal, and 500 μM ubiquitin. Reactions were resolved by SDS-PAGE and stained with Coomassie prior to gel excision. The protein bands were cut from an SDS-PAGE gel and cut into ~1-mm cubes. The gel bands were washed in 200 μl of deionized water for 5 min, followed by 200 μl of 100 mM ammonium bicarbonate/acetonitrile (1:1) for 10 min, and finally 200 μl of acetonitrile for 5 min. The acetonitrile was discarded, and the gel bands were dried in a speed-vac for 10 min. The gel pieces were rehydrated with 70 μl of 10 mM DTT in 100 mM ammonium bicarbonate and incubated for 1 h at 56 °C. The samples were allowed to cool to room temperature, after which 100 μl of 55 mM iodoacetamide in 100 mM ammonium bicarbonate was added and the samples were incubated at room temperature in the dark for 60 min. Following incubation, the gel slices were again washed as described above. The gel slices were dried and rehydrated with 50 μl of trypsin at 50 ng/μl in 45 mM ammonium bicarbonate and 10% acetonitrile on ice for 30 min. The gel pieces were covered with an additional 25 μl of 45 mM ammonium bicarbonate and 10% acetonitrile, and incubated at 37 °C for 19 h. The digested peptides were extracted twice with 70 μl of 50% acetonitrile, 5% formic acid (vortexed 30 min and sonicated 10 min) and once with 70 μl of 90% acetonitrile, 5% formic acid. Extracts from each sample were combined and lyophilized.

The lyophilized in-gel tryptic digest samples were reconstructed in 20 μl of nanopure water with 0.5% formic acid for nanoLC-ESI-MS/MS analysis, which was carried out by a LTQ-Orbitrap Velos mass spectrometer (Thermo-Fisher Scientific) equipped with a CorConNeX nano ion source device (CorSolutions LLC). The Orbitrap was interfaced with a nano HPLC carried out by an UltiMate3000 UPLC system (Dionex). The gel extracted peptide samples (2–4 μl) were injected onto a PepMap C18 trap column-nano Viper (5 μm, 100 μm × 2 cm, Thermo Dionex) at 20 μl/min flow rate for online desalting and then separated on a PepMap C18 RP nanocolumn (3 μm, 75 μm × 15 cm, Thermo Dionex) which was installed in the “Plug and Play” device with a 10-μm spray emitter (NewObjective). The peptides were then eluted with a 90-min gradient of 5% to 38% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min.

The Orbitrap Velos was operated in positive ion mode with nanospray voltage set at 1.5 kV and source temperature at 275 °C. Internal calibration was performed with the background ion signal at m/z 445.120025 as the lock mass. The instrument was operated in parallel data-dependent acquisition mode using FT mass analyzer for one survey MS scan for precursor ions followed by MS/MS scans on top 7 highest intensity peaks with multiple charged ions above a threshold ion count of 7500 in both LTQ mass analyzer and high-energy collision dissociation (HCD)-based FT mass analyzer at 7,500 resolution. Dynamic exclusion parameters were set at repeat count 1 with a 15-s repeat duration, exclusion list size of 500, 30-s exclusion duration, and ≥10 ppm exclusion mass width. HCD parameters were set at the following values: isolation width of 2.0 m/z, normalized collision energy of 35%, activation Q at 0.25, and activation time of 0.1 ms. All data were acquired using Xcalibur operation software (version 2.1, Thermo-Fisher Scientific).

All MS and MS/MS raw spectra were processed and searched using Proteome Discoverer 1.3 (PD1.3, Thermo-Fisher Scientific) against databases downloaded from the NCBI database. The database search was performed with two-missed cleavage site by trypsin allowed. The peptide tolerance was set to 10 ppm, and MS/MS tolerance was set to 0.8 Da for collision-
induced dissociation and 0.05 Da for HCD. A fixed carbamidomethyl modification of cysteine, variable modifications on methionine oxidation, and ubiquitin modification of lysine were set. The peptides with low confidence score (with an Xcorr score < 2 for doubly charged ion and < 2.7 for triply charged ion) defined by PD1.3 were filtered out, and the remaining peptides were considered for the peptide identification with possible ubiquitination determinations. All MS/MS spectra for possibly identified ubiquitination peptides from initial database searching were manually inspected and validated using both PD (version 1.3) and Xcalibur (version 2.1) software.

Cell Culture, Transfection, and Lysate Preparation—All cell lines were obtained from ATCC and cultured in standard medium at 37 °C with 5% CO₂. HEK293T and COS-7 cells were cultured in DMEM with 10% heat inactivated FBS and 1% antibiotic-antimycotic (Cellgro). BHK21 cells were cultured in EMEM with 10% heat-inactivated FBS and 1% antibiotic-antimycotic (Cellgro). Cells were transfected in six-well dishes at 60–80% confluency with 2 μg of total plasmid DNA (293T and BHK21) or 1 μg of total plasmid DNA (COS7) using empty pcDNA3 plasmid to balance all transfections. For jetPRIME® transfection (Polyplus Transfection), a 1:2 (293T) or 1:3 (BHK21 and COS7) ratio of jetPRIME® to DNA was used (w/v), and at 4 h post-transfection, the growth medium was refreshed. At 24 h post-transfection, cells were harvested by trypsinization, washed with PBS, and frozen at −20 °C until analyzed by
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In vitro ubiquitination of β-gal by engineered ubiquibodies. a, in vitro ubiquitination of β-gal in the presence of R4-uAb. At the indicated times, reactions were stopped by boiling and immunoblotted with anti-β-gal antibodies. b, ubiquitination of β-gal was evaluated in the presence (+) or absence (−) of each ubiquitin pathway component, namely ubiquitin (Ub), E1, E2, and R4-uAb as E3. Controls included scFv13-R4, CHIPΔTPR, R4-uAbR272A, and D10-uAb. An equivalent amount of total protein was added to each lane. Immunoblots were probed with anti-β-gal, anti-ubiquitin, anti-Lys-48, and anti-His6 antibodies. Protein bands corresponding to β-gal, mono-ubiquitinated β-gal (*), and β-gal-ubiquitin conjugates as well as the molecular weight of the marker bands (MW) are indicated. The results are representative of at least three replicate experiments.

FIGURE 2. In vitro ubiquitination of β-gal by engineered ubiquibodies. a, in vitro ubiquitination of β-gal in the presence of R4-uAb. At the indicated times, reactions were stopped by boiling and immunoblotted with anti-β-gal antibodies. b, ubiquitination of β-gal was evaluated in the presence (+) or absence (−) of each ubiquitin pathway component, namely ubiquitin (Ub), E1, E2, and R4-uAb as E3. Controls included scFv13-R4, CHIPΔTPR, R4-uAbR272A, and D10-uAb. An equivalent amount of total protein was added to each lane. Immunoblots were probed with anti-β-gal, anti-ubiquitin, anti-Lys-48, and anti-His6 antibodies. Protein bands corresponding to β-gal, mono-ubiquitinated β-gal (*), and β-gal-ubiquitin conjugates as well as the molecular weight of the marker bands (MW) are indicated. The results are representative of at least three replicate experiments.
buffer (0.1 M sodium phosphate, pH 7) with E. coli generated in PyMOL using Protein Data Bank code 1DP0. (ubiquitinated) lysine residue. The modified lysine residue is labeled with GG and corresponds to Lys-775 in carbonate) before measuring absorbance at 420 nm. The amount min and were stopped with 125 μl of stop buffer (1 M sodium carbonate) before measuring absorbance at 420 nm. The amount

lysis buffer. Bound proteins were eluted with 250 mM imidazole (50 mM Tris-HCl, pH 7.9, and 50 mM NaCl) and boiled in 2× Laemmli sample buffer for analysis by immunoblotting.

β-Gal Activity Assay—β-Gal activity was determined using a β-gal assay kit (Invitrogen) according to the manufacturer’s instructions for the microtiter plate format. Briefly, cell pellets were resuspended in 1× lysis buffer (0.25 M Tris, pH 8.0) and lysed with three freeze-thaw cycles before clarification at 18,000 × g for 5 min at 4 °C. Then, 2.5 μl of HEK293T lysate (or 10 μl of COS7 or BHK21 lysate) was added to a well containing 50 μl of 1× cleavage buffer (0.1 M sodium phosphate, 10 mM KCl, 1 mM MgSO4, 7H2O, pH 7) with β-mercaptoethanol and 17 μl of o-nitrophenyl-β-D-galactopyranoside (4 mg/ml). Reactions proceeded at 37 °C for 30 min and were stopped with 125 μl of stop buffer (1 M sodium carbonate) before measuring absorbance at 420 nm. The amount of o-nitrophenyl-β-D-galactopyranoside hydrolyzed was calculated using the following formula: nmol o-nitrophenyl-β-D-galactopyranoside hydrolyzed = (A420(1.92 × 105 cm)/(4500 nl/nmol-cm)(1 cm)). Specific activity was determined according to the following formula: specific activity = o-nitrophenyl-β-D-galactopyranoside hydrolyzed/t/mg protein, where t is the reaction time in minutes and mg is the amount of total protein assayed. For each sample, the background activity from untransfected cell lysates was subtracted, and specific activities within biological samples were normalized to cells transfected with β-gal alone.

RESULTS

Remodeling the Substrate Specificity of CHIP Using Intra-bodies—CHIP is a constitutively active E3 ubiquitin ligase, consisting of an N-terminal tetratricopeptide repeat (TPR) domain, a coiled-coil domain, and a C-terminal U-box ligase.
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domain (23). The TPR domain of CHIP binds to the molecular chaperones Hsc70-Hsp70 and Hsp90, facilitating ubiquitination of chaperone-bound client proteins (24). In this fashion, CHIP uses the chaperone as an adaptor to target a variety of substrates for ubiquitination and degradation by the proteasome. In the absence of substrates, CHIP is able to ubiquitinate the chaperones directly (25). This substrate diversity suggested to us that CHIP specificity could be remodeled using antigenspecific DBPs without affecting its E3 ubiquitin ligase activity (Fig. 1a). To test this hypothesis, we removed the entire TPR domain of CHIP (CHIPΔTPR) and replaced it with a scFv intrabody called scFv13-R4, which specifically binds β-galactosidase (β-gal) (Fig. 1b) (4). The resulting R4-uAb fusion was expressed and purified from E. coli cells. Immunoblotting analysis revealed that R4-uAb was expressed as a stable fusion protein, whereas expression of a fusion with the parental scFv13 clone, which was not optimized for intracellular expression (4) could not be detected (Fig. 1c). Hence, intrabodies, but not conventional scFvs, are a suitable format for uAb development. Importantly, purified R4-uAb was observed to bind β-gal as effectively as scFv13-R4 alone (Fig. 1d), indicating that fusion to CHIPΔTPR does not affect antigen-binding activity of the intrabody domain. In contrast, no significant β-gal binding was seen for CHIPΔTPR or a control uAb comprised of the D10 intrabody (D10-uAb), which is specific for the bacteriophage gpD protein (Fig. 1d) (26).

Next, we performed in vitro ubiquitination assays with purified components, including R4-uAb as the E3 enzyme and β-gal as the target (note that β-gal has 20 lysine residues that serve as potential ubiquitin attachment sites). UbCH5a was used as the E2 enzyme because it has previously been shown to function with CHIP in vitro (10). High molecular weight bands corresponding to ubiquitinated β-gal were observed, especially during longer incubation times (Fig. 2a), which was characteristic of CHIP-mediated polyubiquitination of its native targets (10). These results confirm that the CHIPΔTPR domain retained E3 ligase activity in the context of the chimera. Ubiquitination only proceeded when all pathway components were included in the reaction (Fig. 2b), indicating that R4-uAb activity was dependent on a complete ubiquitination pathway. Similar polyubiquitination was detected with an antibody specific for Lys-48-linked polyubiquitin chains (Fig. 2b), confirming the presence of ubiquitin linkages that are known to signal proteasomal degradation (27). Importantly, when reactions were performed with scFv13-R4, CHIPΔTPR, or the nonspecific D10-uAb, there was no detectable ubiquitination of β-gal (Fig. 2b). Likewise, R4-uAbK272A, which carries an R272A substitution known to inhibit E2 binding (28), failed to conjugate ubiquitin (Fig. 2b), even though it was capable of binding β-gal (Fig. 1d).

Identification of Lysine Residues Modified by Anti-β-gal Ubiquibody—We next investigated which lysine residues were ubiquitinated by our engineered R4-uAb fusion. In vitro ubiquitination of β-gal was monitored by SDS-PAGE, and the formation of higher molecular weight species was clearly evident in the region of the gel where ubiquitinated β-gal would be expected to resolve (Fig. 3a). We also detected R4-uAb-ubiquitin conjugates, consistent with earlier reports showing autoubiquitination of CHIP and CHIP fusions (10). However at early time points, these were at lower molecular weight regions of the gel. Therefore, bands on the gel corresponding to ubiquitinated β-gal after 15 min of ubiquitination, were excised, digested with trypsin, and analyzed by liquid chromatography-tandem mass spectrometry.

FIGURE 4. R4-uAb-mediated proteolysis of β-gal in mammalian cells. a, immunoblots of soluble (top panels) or insoluble (bottom panel) fractions prepared from HEK293T cells transfected with pcDNA3-β-gal alone at 0.05 μg of plasmid DNA per well (β-gal only) or co-transfected with pcDNA3-β-gal along with one of the following: pcDNA3-R4-uAb, pcDNA3-D10-uAb, pcDNA3-R4-uAbK272A, or pcDNA3-scFv13-R4 (each transfected at 1.25 μg of plasmid DNA per well). The triangle indicates increasing amounts of pcDNA3-R4-uAb plasmid DNA (0.05, 0.25, 0.75, and 1.25 μg of plasmid DNA per well) used to transfect cells. The percentages of β-gal remaining in each sample were quantitated by densitometry scanning and are indicated. Blots were probed with antibodies specific for β-gal, His6, GAPDH, and Hsp70 as indicated. An equivalent amount of total protein was loaded in each lane, as confirmed by immunoblotting with anti-GAPDH antibodies. The immunoblot results are representative of at least three replicate experiments. b, β-gal activity measured in samples described in a and in Fig. 5. The activity reported for pcDNA3-R4-uAb corresponds to the highest co-transfection level in 293T and the lowest co-transfection level in COS7 and BHK21. Data were normalized to the signal for the β-gal-only control and is expressed as the mean ± S.D. of biological triplicates.
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Peptides corresponding to 80% of the β-gal sequence were identified using Mascot software. Trypsin digestion of a ubiquitinated protein leaves the C terminus of ubiquitin, Gly-Gly, attached to the ubiquitinated lysine residue. Therefore, we searched the MS data for such modification of β-gal lysines and found five modified residues: Lys-348, Lys-518, Lys-662, Lys-774, and Lys-775. All five lysines are solvent accessible (Fig. 3c), consistent with the location of ubiquitin attachment sites on native CHIP substrates Hsp70 and Hsp90 (21). A representative MS/MS spectrum of a β-gal peptide that includes the identified ubiquitination site Lys-775 is depicted in Fig. 3c.

Peptides corresponding to 95% of the ubiquitin sequence were also identified in the MS experiments. Specifically, Lys-6, Lys-11, Lys-48, and Lys-63 residues were found to be ubiquitinated in peptides isolated from our high molecular weight samples, suggesting these chain linkages in the polyubiquitation of β-gal by R4-uAb (data not shown). These same chain linkages were observed previously on natural substrates that had been ubiquitinated by full-length CHIP in vitro (21). It is worth noting that although Lys-48-linked chains are considered the canonical linkage associated with targeting proteins for proteasomal degradation, all linkages identified here, including Lys-6, Lys-11, and Lys-63, have been implicated as targeting signals for the 26S proteasome (29, 30).

Ectopic Expression of R4-uAb Fusion Mediates Proteasomal Degradation—We next investigated whether R4-uAb-mediated ubiquitination would result in β-gal depletions by the UPP in mammalian cells. First, HEK293T cells were transiently co-transfected with pcDNA3-based plasmids encoding E. coli β-gal and the R4-uAb, where each construct was under control of the strong human CMV promoter. Next, cellular β-gal levels were measured by immunoblotting and by determining β-gal activity 24 h post-transfection. When HEK293T cells were co-transfected with pcDNA3-β-gal and increasing amounts of pcDNA3-R4-uAb, the β-gal levels were systematically reduced to as low as 3% of the steady-state levels measured in cells transfected with only the pcDNA3-β-gal plasmid (Fig. 4a). In contrast, no reduction in β-gal expression or activity was observed following co-transfection with the pcDNA3-D10-uAb or pcDNA3-scFv13-R4 plasmids (Fig. 4, a and b). Interestingly, cells co-transfected with R4-uAb exhibited an intermediate level of β-gal expression (Fig. 4a), indicating that this point mutation in the U-box domain of CHIP inhibits some but not all E2 interactions in vivo. Importantly, the levels of a housekeeping protein, GAPDH, and a native binding partner of full-
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length CHIP, Hsp70, were not affected by co-transfections (Fig. 4a). Evaluation of insoluble fractions confirmed that β-gal depletion was not due to partitioning into aggresomes, aggresome-like induced structures (31, 32), or inclusion bodies (Fig. 4a). Similar silencing results for co-transfection of pcDNA3-β-gal and pcDNA3-R4-uAb were obtained in BHK21 and COS-7 cells (Fig. 4b and 5), indicating that target protein knock-out by engineered ubiquibodies is transferable between different cell lines.

To confirm that R4-uAb specifically binds and ubiquitinates β-gal in vivo, interactions involving β-gal were determined using a pulldown assay. HEK293T cells were transiently transfected with pcDNA3-R4-uAb or pcDNA3-β-gal alone, or co-transfected with pcDNA3-β-gal and pcDNA3-R4-uAb, pcDNA3-scFv13-R4, or pcDNA3-D10-uAb, which each contained C-terminal His6 tags. As expected, both R4-uAb and scFv13-R4, but not D10-uAb, co-precipitated β-gal (Fig. 6). Furthermore, high molecular weight proteins co-precipitated by R4-uAb were observed to cross-react with an anti-ubiquitin antibody (Fig. 6), suggesting that ubiquitinated β-gal was present in cells co-transfected with pcDNA3-R4-uAb. These results suggest that β-gal depletion in mammalian cells results from specific target binding and ubiquitination by the engineered R4-uAb.

An FN3-based Ubiquibody Promotes Degradation of Malto-ose-binding Protein—To demonstrate the generality and modularity of our approach, we sought to create a new ubiquibody against a different target antigen by remodeling the substrate specificity of CHIP with a small antibody mimetic domain known as a monobody. Specifically, we fused an FN3 monobody called YS1 (MBP-74) that is specific for E. coli MBP (33) to the N terminus of CHIPTPR, resulting in the ubiquibody YS1-uAb. Co-transfection of HEK293T cells with pcDNA3-MBP and increasing amounts of pcDNA3-YS1-uAb systematically reduced MBP levels to as low as 7% of the steady-state levels measured in cells transfected with only the pcDNA3-MBP plasmid (Fig. 7, a and b). For the lowest level of MBP gene dosage (0.01 μg of plasmid DNA per well), YS1-uAb-mediated silencing was equally effective over all dosages of ubiquibody plasmid DNA (Fig. 7a). As the MBP dosage was increased (0.05 and 0.1 μg of plasmid DNA per well), the extent of knockdown correlated linearly with ubiquibody gene dosage, ranging from 80% to below 10% of the target protein remaining in cells (Fig. 7c). Thus, variable degrees of knockdown were achieved by simply changing the ratio of pcDNA3-MBP plasmid to pcDNA3-YS1-uAb plasmid, demonstrating the potential for tuning ubiquibody knockdown based on gene dosage. In contrast, the Hsp70 levels were unchanged in the presence of YS1-uAb (Fig. 7), confirming that YS1-uAb-mediated proteolysis is highly specific and CHIPΔTPR-dependent.

**DISCUSSION**

We have shown that engineered ubiquibodies are a generalizable platform for directing otherwise stable proteins to the UPP for degradation. Ubiquibodies exploit the modular architecture of human CHIP, an E3 ubiquitin ligase with exquisite structural flexibility that accommodates substrates with different sizes and structures (10). Specifically, we swapped the N-terminal TPR domain of CHIP, which is responsible for
FIGURE 7. **YS1-uAb-mediated proteolysis of MBP in mammalian cells.** a and b, representative immunoblots of soluble or insoluble (bottom panel of a) fractions prepared from HEK293T cells transfected with pcDNA3-MBP alone (MBP only at 0.01 (a) or 0.1 μg (b) of plasmid DNA per well) or co-transfected with pcDNA3-MBP along with one of the following: pcDNA3-YS1-uAb, pcDNA3-YS1, pcDNA3-R4-uAb, or pcDNA3-D10-uAb (each transfected at 1.75 μg of plasmid DNA per well). The triangles indicate increasing amounts of pcDNA3-YS1-uAb plasmid DNA (0.25, 0.75, 1.25, and 1.75 μg of plasmid DNA per well in a and 0.5, 1.5, and 1.75 μg of plasmid DNA per well in b) used to transfect cells. The percentages of MBP remaining in each sample were quantitated by densitometry scanning and are indicated. Blots were probed with antibodies specific for MBP, His₆, Hsp70, and GAPDH as indicated. An equivalent amount of total protein was loaded in each lane, as confirmed by immunoblotting with anti-GAPDH antibodies. The immunoblot results are representative of at least two replicate experiments. c, scatter plot of densitometry analyses from five independent MBP knockdown experiments in HEK293T cells, where the level of MBP transfection was 0.05 or 0.1 μg of plasmid DNA per well as indicated. Linear regression analysis was performed on the data corresponding to the two different MBP transfection levels and R² values are shown.
Selective Protein Knock-out Using Engineered Ubiquibodies

native substrate binding, for a DBP of known specificity. The resulting ubiquibodies were highly specific for their intended targets while avoiding Hsp70, a natural binding partner and potential substrate of wild-type CHIP. Owing to the catalytic nature of ubiquitination, a single ubiquibody molecule has the potential for elimination of numerous copies of its target protein. This represents a major advantage over target inactivation using binding proteins alone, which require a one-to-one stoichiometric ratio (or greater) with their target because there are no elimination pathways for binding protein-target complexes. Moreover, ubiquibodies based on CHIP do not rely on other subunits for their functionality, making them self-sufficient compared with targeted proteolysis methods that exploit multimeric SCF-type E3 ligases (12–14).

In this work, we attained significant knockdown in three different cell lines (HEK293T, BHK21, and COS7) using transient co-transfection to deliver target and ubiquibody proteins. The extent of silencing correlated fairly well with the dosage of the ubiquibody plasmid DNA, with knockdown ranging from 80% to as little as 3% of the target protein remaining in cells. Nevertheless, under the conditions tested here, complete target ablation was not observed. We speculate that this was due to (i) poor transfection efficiency associated with transient co-transfection (12) and (ii) the relatively high level of plasmid-based target protein expression. We are optimistic that future silencing of endogenous targets may result in complete target ablation given the lower expression level for most chromosomally encoded gene products as well as the potential for improved transfection efficiency when the target protein plasmid is omitted. Transfection efficiency could be even further improved by infection with different doses of recombinant adenoviruses expressing ubiquibody genes. Along similar lines, better control of knockdown might be possible through the use of tightly regulated, externally inducible promoters to activate ubiquibody expression. Alternatively, fine-tuning of silencing activity might be possible through the creation of ubiquibody “switches” whereby target binding or ubiquitin conjugation activity is engineered to be dependent on a small molecule ligand (34) or even light (35). Such allosteric ubiquibodies could potentially promote faster degradation because their response would only require changes in proteins already present in the cell and would not be slowed by having to wait for transcription/translation.

It should be pointed out that our approach was reminiscent of a previous study in which targeted silencing of the oncoprotein c-Myc was accomplished by replacing the TPR domain of CHIP with Max, a binding partner of c-Myc (16). In a notable departure from this earlier work, we devised a more general strategy for depleting target proteins that does not require knowledge of the interaction network of a protein but instead leverages the growing repertoire of pre-existing DBPs or the availability of techniques for their rapid isolation (e.g. phage display, cell surface display). In light of this design, a notable constraint is the requirement that the DBP fold properly in the intended subcellular compartment (e.g. cytoplasm). Indeed, when a traditional scFv antibody fragment was fused to CHIPΔTPR, no detectable expression was observed. This is because correct folding and function of a prototypical scFv requires disulfide bond formation (4), which is disfavored in the reducing cytoplasmic compartment. As demonstrated here, affinity domains that are engineered to function in the absence of their native disulfide bonds (e.g. scFv-based intrabodies) or that do not contain disulfide bonds such as small, highly stable antibody mimics (e.g. FN3, DAPRins, etc.) can easily overcome this limitation. Another issue worth considering is the known autoubiquitination of CHIP (10), which could result in unwanted depletion of the ubiquibody. However, even though polyubiquitination of R4-uAb was consistently observed in vitro, stable accumulation of all ubiquibodies was observed in mammalian cells, with expression levels increasing in a plasmid dose-dependent fashion. Nonetheless, it might be possible to eliminate autoubiquitination by mutating acceptor lysines in CHIP to residues that do not adversely affect the ability of CHIP to interact with cognate E2 and transfer ubiquitin to desired substrates.

Domain swapping with different DBPs (e.g. scFv intrabody, FN3 monobody) confirmed the striking conformational flexibility of CHIP in ubiquitin transfer (10), targeting multiple acceptor lysines on two structurally distinct substrates with varied distances and of continuously increasing size (due to the growing ubiquitin chain). These results also confirm the potential of CHIP for customizable target degradation. Indeed, given the plethora of existing DBPs against known cellular targets and the availability of robust technologies for on-demand isolation of new DBPs that function inside cells (26, 36, 37), ubiquibodies are likely to become a powerful tool for reverse genetics. In addition to the wide array of endogenous proteins that can be targeted with newly designed ubiquibodies, the existing R4-uAb construct described here could be used in conjunction with β-gal protein trapping (38) to silence virtually any β-gal-tagged protein expressed from its endogenous loci in cultured cells and whole organisms. Furthermore, because ubiquibodies operate at the post-translational level and their DBP domains can be created to bind specific protein conformations or post-translational modifications such as phosphorylation (39), the ubiquibody technique has the potential for depleting certain protein isoforms while sparing others. This is significant because post-translational modification-specific silencing of proteins is not possible using current knockdown methods that function at the level of DNA or RNA. Thus, ubiquibodies offer a simple, reproducible, and customizable technique for selectively and controllably accelerating the turnover of numerous proteins and protein isoforms in somatic cells.

Acknowledgments—We thank Cam Patterson, Pierre Martineau, Andreas Plückthun, and Shohei Koide for kindly providing plasmids tagged protein expressed from its endogenous loci in cultured cells and whole organisms. Furthermore, because ubiquibodies operate at the post-translational level and their DBP domains can be created to bind specific protein conformations or post-translational modifications such as phosphorylation (39), the ubiquibody technique has the potential for depleting certain protein isoforms while sparing others. This is significant because post-translational modification-specific silencing of proteins is not possible using current knockdown methods that function at the level of DNA or RNA. Thus, ubiquibodies offer a simple, reproducible, and customizable technique for selectively and controllably accelerating the turnover of numerous proteins and protein isoforms in somatic cells.

REFERENCES


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