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Redirecting the specificity of ubiquitination by modifying ubiquitin-conjugating enzymes

(protein degradation/gene knockout/phage display)

MARK M. GOSINK AND RICHARD D. VIERSTRA*

Department of Horticulture, University of Wisconsin, Madison, WI 53706

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ABSTRACT **Depletion of specific cellular proteins is a powerful tool in biological research and has many medical and agricultural benefits. In contrast to genetic methods currently available to attenuate protein levels, we describe an alternative approach that redirects the ubiquitin-dependent proteolytic pathway to facilitate specific proteolytic removal. Degradation via the ubiquitin pathway requires the prior attachment of multiple ubiquitins to the target protein. This attachment is accomplished, in part, by a family of enzymes designated E2s (or ubiquitin-conjugating enzymes), some of which use domains near their C termini for target recognition. Here, we demonstrate that E2 target recognition can be redefined by engineering E2s to contain appropriate protein-binding peptides fused to their C termini. In five dissimilar examples, chimeric E2s were created that recognized and ubiquitinated their respective binding partners with high specificity. We also show that ubiquitination of one protein targeted by this method led to its ATP-dependent degradation *in vitro*. Thus, by exploiting interacting domains derived from natural and synthetic ligands, it may be possible to design E2s capable of directing the selective removal of many intracellular proteins.**

The selective depletion of intracellular proteins is a powerful approach for dissecting cellular processes and recently has been exploited in a number of medical and agricultural applications (1). Most techniques involve direct or indirect manipulation of the targeted gene or its expression and include such commonly used methods as gene disruption, suppression with antisense RNA, and gene silencing (1, 2). In only a few cases have proteolytic methods for removing the protein product been exploited, even though this catabolic step can be rapid and occurs with high specificity (3, 4). In one example, the attachment of destabilizing peptide domains proved effective but is inherently unsuitable for removing endogenous unmodified targets (4).

We present here a conceptually new proteolytic approach for reducing the levels of specific eukaryotic proteins by redirecting the ubiquitin-dependent proteolytic pathway to recognize and break down otherwise stable proteins. In the pathway, ubiquitin functions as a reusable signal for proteolysis (5, 6). Via an ATP-dependent cascade of reactions, proteins targeted for degradation are first covalently tagged with chains of multiple ubiquitins; the resulting ubiquitin–protein conjugates then are selectively recognized by the ATP-dependent 26S proteasome, which degrades the target protein and releases ubiquitin intact. The pathway is responsible for degrading most abnormal proteins and many short-lived cell regulators (5, 6).

Within the pathway, E2s (ubiquitin-conjugating enzymes) play a prominent role in selectivity by helping recognize appropriate targets for ubiquitination (5–7). They function by facilitating the transfer of ATP-activated ubiquitin from E1 (ubiquitin-activating enzyme) to the target protein. This transfer begins with formation of a ubiquitin–E2 intermediate where the C terminus of ubiquitin is bound via a thiol ester bond to a specific cysteine within the E2 and ends with synthesis of a ubiquitin–protein conjugate where the C terminus of ubiquitin is linked via an isopeptide bond to a free lysl ε-amino group within the target.

E2s comprise a complex family of enzymes that differ in amino acid sequence, molecular mass, and target specificity (6, 7). All contain a conserved core domain of ≈150 aa that includes the active-site cysteine. Some E2s consist of the core alone, whereas others contain additional sequences within and/or extending beyond the N or C terminus of the core domain (6, 7). Although many E2s require an additional factor, E3 or ubiquitin protein ligase, for target recognition and ubiquitin transfer, *in vitro* and *in vivo* studies suggest that some E2s interact directly with their targets, possibly through these additional sequences extending beyond the core (6, 7). In one example, Sullivan and Vierstra (8) demonstrated that a C-terminal extension of one E2 could be the sole determinant in correct substrate recognition *in vitro* and could be functionally transferred to another E2.

Given the pivotal role of E2s in correct substrate recognition, we examined whether the ubiquitin pathway could be redirected to recognize new targets by appending appropriate protein-binding domains onto the C termini of E2s. Interaction between the chimeric E2 and the target protein would enhance ubiquitination of the target, ultimately leading to its degradation by the 26S proteasome. To test this approach, a variety of protein binding domains were fused by recombinant DNA methods onto the C termini of two dissimilar E2s. We show here that such simple modifications can be sufficient to redirect ubiquitination to specific substrates and, in the one case tested, can lead to selective ATP-dependent degradation *in vitro*.

MATERIALS AND METHODS

Materials. Wheat germ extract (9), rabbit reticulocyte lysate (10), and crude membrane preparations of A431 epidermoid carcinoma cells (11) were prepared as described. Reduced carboxymethylated ^{125}I-labeled IgGs (^{125}I-IgGs) were synthesized as described (12) using Iodo-Beads (Pierce) to ^{125}I-label mouse IgGs (Sigma) and iodoacetamide for alkylation.

Construction of Chimeric E2s. All manipulations were performed with the *AtUBC1* and *TaUBC4* genes inserted in the pET3a expression vector (8). *Xho* I sites were engineered by site-directed mutagenesis into the 3′ ends of pET-AtUBC1 and pET-TaUBC4 with the oligonucleotides GCAAAGCTGGACTGCTCTCGAGTAGTAGTTTGTTGTAAGCG and GG-

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Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; TGFα, type α transforming growth factor; EGFR, epidermal growth factor receptor.

*To whom reprint requests should be addressed.

CCACGCAGACCCTCTCGAGTAGGATGGATGCAAGG, respectively. The annealed oligonucleotides, TCGAGGAGCAGAAGCTGATCAGCGAGGAGGACCTGTAAC and TCGAGTTACAGGTCCTCCTCGCTGATCAGCTTCTGCTCC, encoding the c-MYC epitope (EQKLISEEDL) recognized by the monoclonal antibody 9E10 (13) were ligated into the *Xho* I site of the appropriate plasmids to create pET-AtUBC1-c-myc and pET-TaUBC4-c-myc. pET-AtUBC1-spacer-c-myc was generated by ligating the annealed oligonucleotides TCGAACCACCAGTCGACGCAGCAGCAGCAGCACTCGAGT and TCGAACTCGAGTGCTGCTGCTGCTGCGTCGACTGGTGGT into the *Xho* I site of pET-AtUBC1, creating pET-AtUBC1-spacer. The annealed oligonucleotides encoding the c-Myc epitope (see above) then were introduced into an *Xho* I site located at the 3′ end of the spacer. To construct pET-TaUBC4-TGFα, the coding region for the human type α transforming growth factor (TGF-α) (aa 41–89) was amplified by PCR from pCMVTGF-Neo (14) with the oligonucleotides CCCGCCCGTGGCTGCACTCGAGGTGTCCCATTTTAATGACTGCCC and GGCCTGCTTCTTCTGGCTGGCGTCGACCTAGGCCAGGAGGTCCGCATGC. The primers added a stop codon after the 89th codon of TGF-α and *Xho* I and *Sal* I sites to the 5′ and 3′ ends, respectively. The PCR fragment was digested with *Xho* I/*Sal* I and ligated directly into the *Xho* I site of pET-TaUBC4. The coding region for the immunoglobulin-binding region D [aa 90–153 (15)] was PCR amplified from genomic *Staphylococcus aureus* DNA with oligonucleotides CTTAATGACCTCGAGGCTCCAAAAGCTGATGCGCAAC and GTTGAAATTCTCGAGTTATTTCGGTGCTTGAGATTCG designed so that *Xho* I sites were added to the 5′ and the 3′ ends of the amplified fragment and a stop codon followed the 153rd codon. The coding region for the immunoglobulin-binding regions DABC [aa 90–327 (15)] was amplified with oligonucleotides CTTAATGACCTCGAGGCTCCAAAAGCTGATGCGCAAC and GCTTGTTATTCTCGAGTTATTTTGGTGCTT designed so that *Xho* I sites were added to the 5′ and the 3′ ends of the amplified fragment and a stop codon followed the 327 codon. pET-AtUBC1-spacer-protein A_{DABC} was constructed by ligating this fragment into pET-AtUBC1-spacer digested with *Xho* I. pET-AtUBC1-spacer-S_{pep} was constructed using PCR to amplify the 15-aa S-peptide fragment (16) from pET-29c (Novagen) with the oligonucleotides GATCTCGAGATGAAAGAAACCGCTGC and CCCGGATCCTAGCTGTCCATGTGC. The resulting fragment was digested with *Xho* I/*Bam*HI and ligated into pET-AtUBC1-spacer similarly digested. pET-AtUBC1-spacer-S_{ant} was created by ligating the annealed oligonucleotides TCGACTACAACTTCGAAGTTCTGTGAG and GATCCTCACAGAACTTCGAAGTTGTAG into pET-AtUBC1-spacer digested with *Xho* I/*Bam*HI.

Production and Assay of Chimeric E2s. E2 proteins were synthesized in *Escherichia coli* as described (8). For protein degradation assays, E2s were partially purified by DE-52 chromatography using a step elution of 100 mM KCl in 50 mM Tris·HCl, pH 7.2 (4°C)/1 mM dithiothreitol (DTT). Thiol ester and conjugation assays were performed as described (8), except that thiol ester reactions proceeded for 5 min at room temperature and the conjugation reaction mixtures were incubated for 2 h at 30°C. The amount of E2 protein used in each conjugation reaction was normalized so that equivalent thiol ester-forming activities were used. Where indicated, conjugation products of IgGs were partially purified by precipitation with protein A-agarose beads (Sigma).

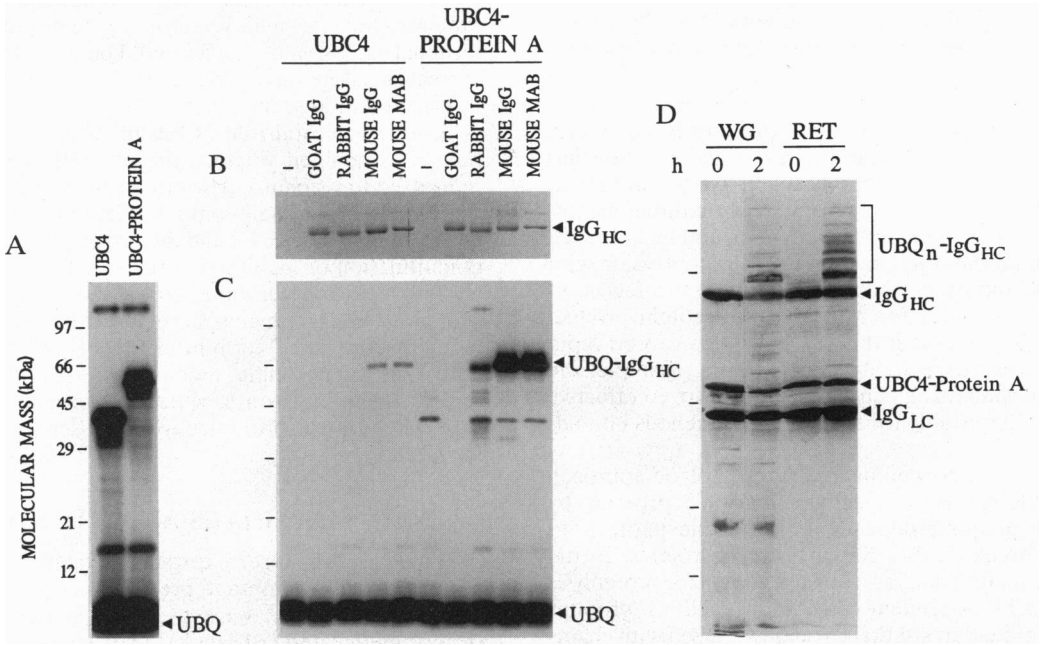

FIG. 1. Engineering an E2 to recognize and conjugate ubiquitin to various IgGs by exploiting the binding affinity of protein A. TaUBC4 protein was modified to contain the immunoglobulin-binding D domain of protein A fused directly to its C terminus (15). (*A*) Ubiquitin-accepting activities of TaUBC4 and TaUBC4-protein A_D were determined by thiol ester assay with ^{125}I-ubiquitin. An equal amount of E2 (determined by Coomassie staining) was added to each reaction mixture. (*B* and *C*) Ubiquitin conjugation reactions using either TaUBC4 or TaUBC4-protein A_D and various IgGs as substrates. ^{125}I-ubiquitin conjugation reactions were performed with equal amounts of active E2 (determined by thiol ester assay). Products were partially purified by precipitation with protein A-agarose beads and subjected to SDS/PAGE. (*B*) Coomassie-stained gel of IgGs precipitated from the conjugation reactions with protein A-agarose beads. Amount of IgG added to each conjugation reaction was normalized so that equivalent amounts of each IgG were precipitated. (*C*) Autoradiograph of ^{125}I-ubiquitin–IgG conjugates. The following IgGs were used: –, no added IgG; GOAT IgG, goat IgG at 5 mg/ml; RABBIT IgG, rabbit IgG at 0.75 mg/ml; MOUSE IgG, mouse IgG at 0.31 mg/ml; MOUSE MAB, mouse monoclonal IgG at 0.31 mg/ml. (*D*) Ubiquitin conjugation to mouse IgG by TaUBC4–protein A_D in the presence of either wheat germ extract (WG) at 6 mg/ml or rabbit reticulocyte lysate (RET) at 24 mg/ml. Conjugation reactions were performed in 50 mM Tris·HCl (pH 9.0) and unlabeled ubiquitin. At the times indicated, the reaction mixtures were quenched and subjected to SDS/PAGE and immunoblot analysis using alkaline-phosphatase-labeled goat anti-mouse IgGs. Designations indicate positions of unincorporated ubiquitin (UBQ), IgG heavy chain–ubiquitin adducts (UBQ_n–IgG_{HC}), TaUBC4-protein A_D (UBC4–Protein A), and free IgG heavy and light chains (IgG_{HC} and IgG_{LC}).

Protein Degradation Assays. Protein degradation assays (total vol, 80 μl) contained 3.6 mg of rabbit reticulocyte lysate (10), 12 μg of reduced carboxymethylated mouse ^{125}I-IgGs, 10 μg of bovine ubiquitin, 2 μg of E1, 4 μg (total protein) of partially purified E2s, 50 mM Tris·HCl (pH 8.5), 2 mM DTT, and either an ATP-depleting or an ATP-regenerating system (9). After various times of incubation at 37°C, aliquots were removed and protein was precipitated with cold 10% trichloroacetic acid. Radioactivity in the supernatants and pellets (dissolved in 100 μl of 1 M NaOH) was determined by scintillation counting.

RESULTS AND DISCUSSION

In our attempt to engineer an E2 with predefined specificity, coding regions for a number of well-characterized protein ligands were fused to the 3′ ends of coding regions of two E2s, *AtUBC1* from *Arabidopsis thaliana* and *TaUBC4* from wheat (*Triticum aestivum*) (8). The resulting E2s were expressed in *E. coli* and, except where noted, the lysed cells were used as the E2 source without further purification. In our first example, a 64-aa peptide encompassing one of the four immunoglobulin-binding domains (domain D) of *S. aureus* protein A (15) was appended directly to the C terminus of TaUBC4. As shown in Fig. 1*A*, addition of this peptide did not affect the ability of E2 to accept activated ubiquitin from E1. In reaction mixtures containing equivalent amounts of TaUBC4–protein A_D or unmodified TaUBC4 (as determined by protein staining), near equal levels of the E2–ubiquitin thiol ester adduct were synthesized.

Importantly, addition of the protein A_D domain to TaUBC4 increased substantially the ubiquitination of IgGs (Fig. 1 *B* and *C*). In conjugation reaction mixtures containing the chimeric E2, ^{125}I-ubiquitin, E1, and ATP, the heavy chain of both rabbit and mouse IgGs became modified, with a monoubiquitinated species being the predominant product. Conjugation was specific for certain classes of IgGs and reflected the binding specificity of intact protein A (Fig. 1*C*). Ubiquitination was not evident for goat IgGs but was substantial for both rabbit and mouse IgGs in accord with the poor affinity of protein A for goat IgGs and the high affinity of protein A for the rabbit and mouse counterparts (17).

Other types of antibody–antigen interactions were also successful in modifying E2 specificity. For example, unmodified TaUBC4 would effectively conjugate ubiquitin to the heavy chains of polyclonal immunoglobulins that bind TaUBC4 (data not shown). Likewise, TaUBC4 containing the 10-aa c-Myc epitope [EQKLISEEDL (13)] fused directly to its C terminus would conjugate ubiquitin to the heavy chain of the corresponding 9E10 IgG (13) through interaction of the epitope with the Fv region (Fig. 2 *Left*). No conjugates were formed when unmodified TaUBC4 was used, when a nonbinding monoclonal IgG (S3) was substituted for 9E10, or when a peptide containing the c-Myc epitope was added in excess (Fig. 2 *Left*). In the latter control, we observed a ubiquitin adduct of the free c-Myc peptide presumably by the acidic C-terminal domain of TaUBC4 interacting with the basic residues of the c-Myc peptide used.

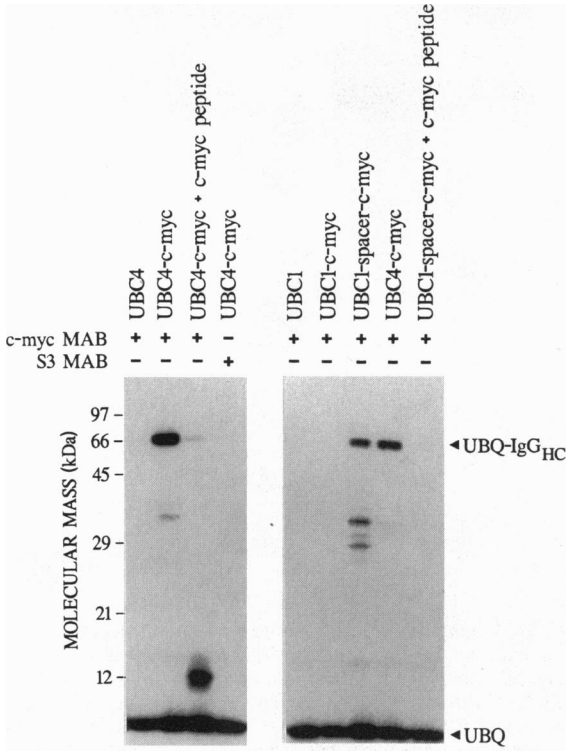

FIG. 2. Engineering E2s to specifically recognize and conjugate ubiquitin to an IgG by exploiting an epitope–IgG interaction. AtUBC1 and TaUBC4 were modified to contain the 10-aa c-Myc epitope [EQKLISEEDL (13)] appended directly to the C terminus of TaUBC4 (UBC4–c-myc) and AtUBC1 (UBC1–c-myc) or indirectly through a 10-aa spacer to AtUBC1 (UBC1–spacer–c-myc). ^{125}I-ubiquitin conjugation reactions used either the anti-c-Myc monoclonal IgG, 9E10 (c-myc MAB), or an unrelated nonbinding monoclonal IgG (S3 MAB) at 50.0 μg/ml. Where indicated, a 32-aa peptide containing the c-Myc epitope (Oncogene Science) was added to 5.0 μg/ml. Reaction products were partially purified by precipitation with protein A-agarose beads and subjected to SDS/PAGE and autoradiography. (*Left*) Ubiquitin conjugation reactions using TaUBC4 and TaUBC4–c-myc. (*Right*) Ubiquitin conjugation reactions of AtUBC1, AtUBC1–c-myc, and AtUBC1–spacer–c-myc compared with TaUBC4–c-myc. Arrowheads indicate positions of monoclonal IgG heavy chain–ubiquitin adduct (UBQ–IgG$_{HC}$) and unincorporated ^{125}I-ubiquitin (UBQ).

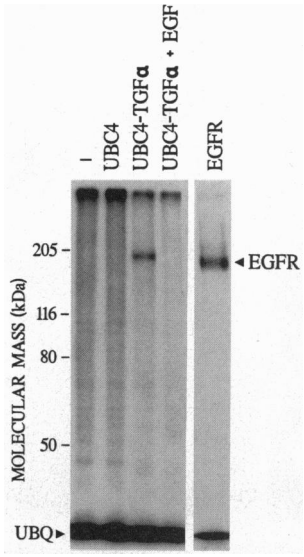

FIG. 3. Engineering an E2 to recognize and conjugate ubiquitin to the EGFR by exploiting a peptide hormone–receptor interaction. TaUBC4 was modified to contain TGF-α attached to its C terminus (14). ^{125}I-ubiquitin conjugation reactions were performed with a crude membrane preparation from human epidermal cells at 0.48 mg of protein per ml. After the reactions, membranes were collected by centrifugation and resuspended in hot SDS/PAGE sample buffer, and the solubilized material was subjected to SDS/PAGE. Ubiquitin conjugation reaction mixtures contained the following: –, no added E2s; UBC4, TaUBC4; UBC4–TGFα, TaUBC4 fused with TGF-α; UBC4–TGFα + EGF, TaUBC4 fused with TGF-α and free EGF (5 mM). Migration position of ^{32}P-EGFR is indicated for size comparison.

Recognition of the anti-c-Myc IgG 9E10 was not restricted to TaUBC4–c-myc but also was possible with AtUBC1 provided a short spacer (PPVDAAAAAL), designed to extend from the E2 core, was inserted between the E2 and c-Myc epitope. When AtUBC1–spacer–c-myc was added to conjugation reaction mixtures containing 9E10 IgG, monoubiquitination of the IgG heavy chain was detected (Fig. 2 *Right*). This adduct was absent when unmodified AtUBC1 or the spacerless AtUBC1–c-myc was used or when excess c-Myc peptide was added to reaction mixtures containing AtUBC1–spacer–c-myc.

Other protein–protein interactions were examined in addition to those using IgGs. In one example, the 49-aa peptide hormone, TGF-α and its binding partner epidermal growth factor receptor (EGFR) were exploited (18). When a TaUBC4–TGF-α fusion was added to crude membrane preparations from human epidermal cells (14) along with ^{125}I-ubiquitin, ATP, and E1, specific monoubiquitination of the EGFR was detected (Fig. 3). The product was not detected in the absence of added E2s, when an equal amount of unmodified TaUBC4 was used, or when an excess of a TGF-α homolog EGF (18) was added as a competitor (Fig. 4). These results highlight the specificity and sensitivity of the E2–protein interaction; even though we were unable to detect EGFR among the multitude of other proteins in the membrane preparations by protein staining (data not shown), it was easily detected by ^{125}I-ubiquitin tagging.

As another example, we used the S-protein and S-peptide binding pair of RNase A (16). A 15-aa peptide containing the essential binding elements of S peptide (16) was appended to the C terminus of AtUBC1 through the spacer described above (see Fig. 3). The chimera, AtUBC1–spacer–S_{pep}, was effective in binding ^{125}I-ubiquitin via a thiol ester bond and, when added to conjugation reaction mixtures, specifically ubiquitinated free S protein (Fig. 5). Conjugates were not detected when unmodified AtUBC1 was used instead or when free S peptide was added in excess.

For the chimeric E2s to generate substrates suitable for breakdown by the 26S proteasome, multiubiquitination of the target may be essential (5, 6). However, in all our examples, monoubiquitinated targets were the predominant products. We found that multiubiquitination will occur, provided additional cellular factors are included in the conjugation reaction mixtures. Although mouse IgG heavy chains were only monoubiquitinated by TaUBC4–protein A_D alone, they were conjugated with multiple ubiquitins when either wheat germ extract or rabbit reticulocyte lysate was included in the reaction mixture (Fig. 1*D*). Both of these crude preparations represent rich sources of enzymes involved in ubiquitin conjugation (5, 9). In each case, a ladder of ubiquitin–IgG heavy chain conjugates was detected with size distributions similar to those found with natural ubiquitin pathway substrates (19–21).

Success of this approach will require that the ubiquitinated proteins serve as substrates for degradation by the 26S proteasome (5, 10). To demonstrate this proteolysis *in vitro*,

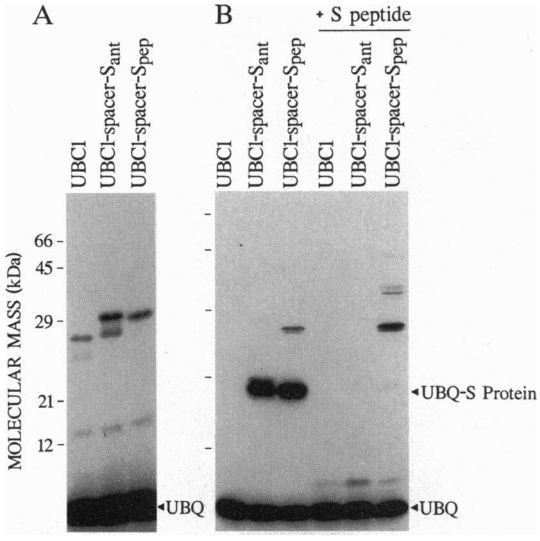

FIG. 4. Engineering an E2 to recognize and conjugate ubiquitin to RNase A S protein by exploiting as ligands either the S peptide or an antagonist peptide identified from a phage-display library (16). AtUBC1 was modified to contain either a 15-aa fragment of the S peptide (UBC1–spacer–S_{pep}) or the S-peptide antagonist YNFEVL (UBC1–spacer–S_{ant}) linked to the C terminus through a 10-aa spacer. (*A*) Activities of AtUBC1, AtUBC1–spacer–S_{pep}, and AtUBC1–spacer–S_{ant} were determined by thiol ester assay using ^{125}I-ubiquitin. (*B*) ^{125}I-ubiquitin conjugation reactions using the AtUBC1 derivatives and S protein as the target. Reactions were performed with equal amounts of active E2 (determined by thiol ester assay) and S protein at 200 μg/ml. Products were subjected to SDS/PAGE and autoradiography. Where indicated, conjugation reaction mixtures contained free S peptide (1 mg/ml).

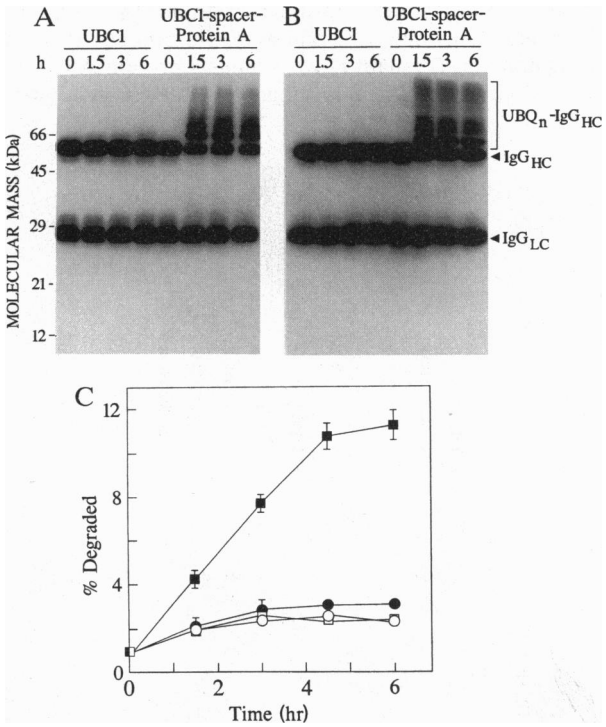

FIG. 5. Ubiquitin conjugation and subsequent degradation of an IgG utilizing an E2–protein A fusion. AtUBC1 was engineered to contain all four immunoglobulin-binding domains (DABC) from protein A (15) attached to the C terminus of E2 through a 10-aa spacer. The ability of AtUBC1 and AtUBC1–spacer–protein A_{DABC} to conjugate ubiquitin to reduced carboxymethylated mouse ^{125}I-IgG was examined in both the absence (*A*) and the presence (*B*) of rabbit reticulocyte lysate. Conjugation reactions were performed in the presence of ATP, nonlabeled ubiquitin, and equal amounts of E2, as determined by thiol ester assay. At the times indicated, the reaction mixtures were quenched and the products were subjected to SDS/PAGE and autoradiography. Designations indicate positions of IgG heavy chain-ubiquitin adducts (UBQ_n–IgG_{HC}), IgG heavy chain (IgG_{HC}), and IgG light chain (IgG_{LC}). (*C*) Degradation of reduced carboxymethylated ^{125}I-IgG by AtUBC1 and AtUBC1–spacer–protein A_{DABC} in the presence of rabbit reticulocyte lysate. Degradation reactions were performed in the presence of excess nonlabeled ubiquitin, equal amounts of either AtUBC1 (○, ●), or AtUBC1–spacer–protein A_{DABC} (□, ■), and either an ATP-regenerating (solid symbols) or an ATP-depleting (open symbols) system. Percentage of ^{125}I-IgG degraded was calculated as percentage total radioactivity soluble in 10% trichloroacetic acid. Each assay was performed in triplicate. Average value ± SD of each time point is shown.

AtUBC1 was fused with a 238-aa peptide encompassing all four immunoglobulin-binding domains of protein A [domains DABC (15)] through the 10-aa spacer PPVDAAAAAL (AtUBC1–spacer–protein A_{DABC}). Because *E. coli* extracts contain an inhibitor of the 26S proteasome (data not shown), we partially purified the E2s before addition to the degradation assay. When incubated with reduced carboxymethylated ^{125}I-IgGs (12), nonradioactive ubiquitin, E1, and ATP, AtUBC1–spacer–protein A_{DABC} ubiquitinated a substantial percentage of the IgG heavy chain; within 90 min as much as 50% of the IgG heavy chains was modified (Fig. 5*A*). While prior reactions using nonradioactive unmodified IgG produced monoubiquitinated products (Fig. 1 *B* and *C*), multiubiquitinated products were evident with the modified ^{125}I-IgGs, even in the absence of extra factors. Conversely, no ubiquitinated products were observed when unmodified AtUBC1 was used (Fig. 5*A*).

Addition of reticulocyte lysate, a rich source of active 26S proteasome (5, 10), to the conjugation reaction mixtures containing ^{125}I-IgG enhanced the multiubiquitination of the IgG heavy chain (compare Fig. 5 *B* and *C*). Importantly, inclusion of the 26S proteasome also led to ATP-dependent breakdown of the ^{125}I-IgGs (Fig. 5*C*). Degradation was observed only in those reaction mixtures containing AtUBC1–spacer–protein A_{DABC} and required ATP, in accordance with the ATP dependence of the 26S proteasome (5). After a 4-h incubation at 37°C, ≈12% of the ^{125}I was found in a trichloroacetic acid-soluble fraction containing small peptides and free amino acids. The percentage of ^{125}I-IgG degraded in an ATP-dependent manner was comparable to that observed with ^{125}I-lysozyme (data not shown), a common substrate for ubiquitin-dependent proteolysis in reticulocyte lysates (10), suggesting that the limited amount of ^{125}I-IgG ultimately degraded reflected the *in vitro* conditions used.

Although the targeted proteolysis approach requires an interacting peptide to recognize the target protein, it need not be limited to those proteins having natural binding partners. We found that an artificial ligand, YNFEVL, identified from a phage display library as a binding antagonist of the S peptide to the RNase A S protein (16), was as effective as S peptide in directing the ubiquitination of S protein. AtUBC1 containing this hexapeptide attached via the spacer to the C terminus (AtUBC–spacer–S_{ant}) was as active in thiol ester assays and in conjugating ubiquitin to S protein as AtUBC1–spacer–S_{pep} (Fig. 4).

Taken together, these results show conceptually that the ubiquitin proteolytic pathway can be engineered to enhance the removal of selected proteins simply by appending appropriate interacting domains onto the C termini of E2s. These binding affinities allow E2s to recognize and ubiquitinate their corresponding binding partners and can ultimately lead to ATP-dependent degradation of the partners *in vitro*. Here we tested a variety of ligand–protein interactions, including protein A–antibody, epitope–antibody, peptide hormone–receptor, and protein subdomains. The ligands ranged in size from 6 to 238 aa and were either natural or artificial. In most cases, appending peptide ligands onto the E2s did little to affect the ubiquitin-accepting activity of the E2, suggesting that there are few constraints on the types of binding interactions that can be exploited. We did find that a spacer peptide [either natural as in TaUBC4 (8) or artificial] separating the E2 core from the ligand may be needed, presumably to facilitate access of the E2 to available accessible lysines within the target. It is noteworthy that all of the substrates we tested became targets of ubiquitination even though they are naturally unaffected by the ubiquitin pathway because of their extracytoplasmic location or prokaryotic origin (14–16). This implies that the only limitations to target choice may be the need for an available lysine(s) and its accessibility to the ubiquitin pathway (5–7).

Given the high conservation of the ubiquitin-dependent proteolytic pathway in all eukaryotes examined (5–7), this targeted proteolytic approach should be applicable in animals, plants, and fungi with E2s derived from various sources. Although our studies employed *in vitro* systems for ubiquitin conjugation and proteolysis, it is likely that the system will function *in vivo* given that the cell-free systems used here were similar to those previously used to successfully reconstitute *in vivo* ubiquitination and degradation (5, 9, 10). If successful *in vivo*, the approach may have several advantages over other methods to eliminate proteins, including its catalytic nature, the fact that neither the target protein nor its corresponding gene requires modification, and its theoretical ability to target for degradation proteins not encoded by the host cell. Predefined ubiquitination may also have potential uses in the biochemical identification of protein-binding partners. As exemplified with EGFR, E2s engineered with appropriate ligands could be used to identify and purify unknown binding partners from a complex mixture by specific labeling with appropriately modified ubiquitin.

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