Substrate Properties of Ubiquitin Carboxyl-Terminally Derived Peptide Probes for Protein Ubiquitination†

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ABSTRACT: Protein ubiquitination is a widespread protein posttranslational modification in eukaryotes that regulates essentially every aspect of cellular processes. The attachment of ubiquitin to a protein substrate is accomplished through an enzymatic cascade involving the actions of an activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). There are more than 600 E3 ligases estimated to exist in the human genome that regulate the targeting specificity of protein ubiquitination. To understand the dynamic role of protein ubiquitination in biological processes, robust tools need to be developed which can be employed to establish the substrate specificity of each of these E3 ligases. In this report, we show that the ubiquitin carboxyl-terminally derived peptide probes can serve as modest ubiquitin surrogates for the ubiquitination pathway. In the E1-catalyzed probe adenylation assay, peptide probe **3** with a RLRGG recognition sequence exhibited the highest activity, with the $k_{cat}/K_{1/2}$ determined to be 1.1×10^4 M^{-1} s^{-1}, roughly 470-fold lower than that of ubiquitin. The rate of transfer from the E1 peptide probe thioesters to E2 showed clear sequence dependency, with peptide probe **4** with an LRLRGG recognition sequence showed the fastest rate ($t_{1/2} = 0.9$ min), essentially identical to that of ubiquitin ($t_{1/2} = 0.8$ min) under our assay conditions. Furthermore, peptide probes **4** and **8** also exhibited the selective, parkin-mediated labeling of tubulins in a semipurified tubulin−parkin complex. Finally, these carboxyl-terminally derived peptide probes were shown to label the ubiquitination substrates in fraction II of the rabbit reticulocyte lysate with an efficiency parallel to their substrate properties. The selective use of these ubiquitin carboxyl-terminally derived peptide probes by the ubiquitination pathway suggests that perhaps more potent peptide ubiquitination probes based on the ubiquitin C-terminal scaffold can be developed through additional structural optimization.

Ubiquitination, the attachment of ubiquitin, a small protein of 76 amino acids, to specific lysine residues on target proteins, regulates a plethora of cellular processes, including proteasome-dependent protein degradation, cell division, nuclear transport, signal transduction, transcription, DNA repair, and apoptosis (*1*–*3*). The ubiquitin conjugation pathway involves successive actions of three enzymes: an activating enzyme (E1),[1] a conjugating enzyme (E2), and a ligase (E3) (*4, 5*) (Figure 1A). An organism typically has a dedicated E1, dozens of E2s, and literally hundreds of E3s. While E1 and ubiquitin are constitutively expressed, target specificity is established primarily through temporospatial expression of unique E3 ligases which recruit a small set of protein substrates in response to specific ubiquitination signals (*6*). Ubiquitination is a highly dynamic and reversible protein posttranslational modification as an estimated 95

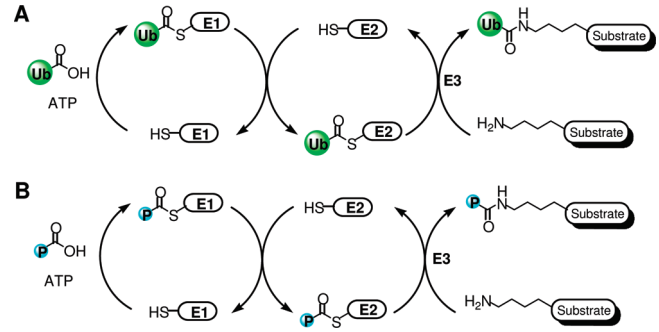

FIGURE 1: Schematic representation of the activation and transfer of ubiquitin and peptide probes by the ubiquitination pathway. Three consecutive enzyme-mediated steps are required for efficient conjugation of either ubiquitin (A) or peptide probes (B) to a protein substrate targeted by the ubiquitination pathway: E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; Ub, ubiquitin; P, peptide probes. ATP is needed for the initial activation of ubiquitin. By pathway B, protein substrates can be rapidly identified through the attachment of peptide probes.

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[1] Abbreviations: Ub, ubiquitin; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; UCH, ubiquitin C-terminal hydrolase; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; DIEA, diisopropylethylamine; DTT, 1,4-dithiothreitol; Ahx, aminohexanoic acid; TCA, trichloroacetic acid.

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putative deubiquitinating enzymes also exist in the human genome to mediate selective removal of ubiquitin from the ubiquitinated substrates (*7*).

To fully understand the role of ubiquitination in cell biology, development, and diseases, it is imperative that the protein substrates for each E3 ligases are identified and their specific ubiquitination sites rigorously established. By taking advantage of the polypeptide nature of ubiquitin, people have used either epitope-tagged ubiquitin, e.g., FLAG-Ub and His-Ub, or biotin-Ub as a modifier in the ubiquitination reaction to enrich the ubiquitinated substrates followed by mass spectrometry analysis. For example, Sato and co-workers identified Nucleophosmin/B23 as a candidate substrate for the BRCA1-BARD1 ubiquitin ligase by incubating FLAG-Ub with the immunoprecipitated BRCA1-BARD1 complex followed by trypsin digestion and nano-LC−MS/MS analysis (*8*). Similarly, in the search for BRCA1-BARD1 substrates in the centrosome, Starita and co-workers used biotin-Ub in an incubation reaction mixture containing the centrosome fraction and the recombinant BRCA1-BARD1 ligase complexes and identified γ-tubulin as a substrate (*9*). A genome-wide profiling of protein ubiquitination in *Saccharomyces cerevisiae* has also been carried out in which His_6-Ub was overexpressed in yeast and the ubiquitination substrates were then pulled down from the cell extract with a Ni column and identified by mass spectrometry (*10*). Using this method, 1075 candidate ubiquitination substrates, along with 110 ubiquitination sites, were identified. While the use of genetically coded ubiquitin has achieved great success in the identification of ubiquitination substrates both in vitro and in vivo, the identification of specific ubiquitination sites remains a major challenge because of the lack of an enrichment step after trypsinization (epitope tags fused to the N-terminus of ubiquitin were cleaved during the digestion). In addition, overexpression of tagged ubiquitin in cells may also lead to an increased level of proteasome-dependent substrate degradation (*11*).

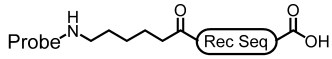
Biochemical and structural studies indicated that ubiquitin is comprised of two functional domains: a nonstructured carboxyl terminus responsible for ubiquitin activation and transfer and a globular region responsible for interactions with diverse downstream effectors (*12–17*). Furthermore, a synthetic carboxyl-terminal fragment of ubiquitin was reported to stimulate the pyrophosphate−ATP exchange, the first step during the ubiquitin activation by E1 enzyme. On the basis of these observations, we hypothesize that the ubiquitin carboxyl terminus could provide a scaffold for peptide probes targeting the ubiquitination pathway. The carboxyl-terminally derived peptide probes can serve as ubiquitin surrogates and label ubiquitination substrates via the ubiquitination pathway (Figure 1B). When appropriate chemical probes, such as biotin, fluorescein, and other small bioorthogonal functionalities (*18, 19*), are employed, these peptide probes can be used not only to identify ubiquitination substrates, including mapping the ubiquitination sites on the protein substrates, but also to study protein dynamics in living cells (e.g., by labeling the ubiquitination substrates with a fluorescent probe) (*20*). In a first step toward these goals, herein we report the biochemical characterizations of the substrate properties of a series of ubiquitin carboxyl-terminally derived peptide probes, including their adenylation by E1, the formation of E1 peptide probe thioesters, the transfer from E1 to E2, the selective labeling of tubulins in the tubulin−parkin complex, and the labeling of ubiquitination substrates in the reticulocyte fraction II cell lysate.

MATERIALS AND METHODS

General Materials and Methods. Human recombinant biotin-N-terminal ubiquitin, rabbit ubiquitin E1, human recombinant UbcH7 (E2), and rabbit reticulocyte fraction II were purchased from Boston Biochem (Boston, MA). The monoclonal anti-α-tubulin (clone DM1A) was purchased from Sigma (St. Louis, MO). The rabbit polyclonal anti-fluorescein IgG (A889) was purchased from Invitrogen (Carlsbad, CA). Fmoc amino acids were purchased from Peptide International (Louisville, KY). Fmoc-Wang resin, HBTU, and HOBt were purchased from Bachem (King of Prussia, PA). Other chemicals were purchased from Aldrich and used without further purification. Tris-glycine SDS−PAGE gels and PVDF membranes were purchased from Invitrogen. [^{32}P]Pyrophosphate tetrasodium salt (86 Ci/mmol) was purchased from Perkin-Elmer (Wellesley, MA). For detection of the biotin-labeled proteins on the PVDF membrane, the VECTASTAIN ABC-AmP detection kit was used (Vector Laboratories, Burlingame, CA) following the manufacturer's instructions. Briefly, the membrane was washed three times for 4 min each in 10 mL of a 1× casein solution at room temperature with gentle shaking and then incubated in 10 mL of a 1× casein blotting solution containing 20 μL each of reagent A and B from the kit for 10 min. The membrane was washed three times for 4 min each in 10 mL of a 1× casein solution, followed by equilibration in 10 mL of 0.1 M Tris buffer (pH 9.5) for 5 min. The excess buffer was removed, and the blot surface was immersed in 3 mL of DuoLux substrate for 5 min under the subdued light. The blot was rinsed briefly in 0.1 M Tris buffer (pH 9.5), and excess buffer was removed by touching the edge of the blot to an absorbent paper. The chemiluminescence was captured by exposing the blot to a Kodak BioMax Light film.

Synthesis of Carboxyl-Terminally Derived Peptide Probes. The peptide probes were assembled from the preloaded Fmoc-Gly Wang resin using a reaction vessel equipped with a polymer filtration frit. The standard Fmoc peptide coupling protocol was followed by employing 3 equiv of Fmoc-AA-OH, 3 equiv of HBTU, 3 equiv of HOBt, 6 equiv of DIEA, and an appropriate amount of DMF to make a 150 mM coupling solution. Coupling reactions were allowed to proceed for 45 min with the exception of that of aminohexanoic acid and biotin, for which the reaction time was extended to 1 h. Peptides were cleaved from the resin using a TFA cleavage cocktail containing 2.5% triisopropylsilane and 2.5% H_2O and precipitated out with the addition of ethyl ether. The precipitate was collected and dried in vacuo. The residues were dissolved in ACN and applied to a Gilson reverse-phase HPLC system running a 30 min 10 to 90% ACN/H_2O gradient with a flow rate of 20 mL/min. The fractions were collected and analyzed by LC−MS, and pure fractions were pooled and lyophilized to afford the peptide probes in powder form. Final structures were confirmed by MS analysis using Finnigan LCQ mass spectrometry running in positive ion mode, and the purities were determined by

Table 1: Structures and Characterization of Peptide Probes by Mass Spectrometry



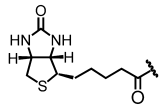
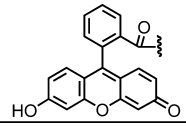
compound	Probe	Recognition Sequence	Calculated [M+H$^+$]	Found [M+H$^+$]
1		RGG	628.8	628.5
2		LRGG	741.9	741.5
3		RLRGG	898.1	897.5
4		LRLRGG	1011.3	1010.6
5		LALRGG	926.2	925.6
6		VLRLRGG	1110.4	1109.8
7		LVLRLRGG	1222.7	1222.8
8		LRLRGG	1142.6	1142.5

Table 2: Characterization of Peptide Adenylation Properties in a Ubiquitin E1-Catalyzed [^{32}P]PP$_i$−ATP Exchange Assay

analogue	$K_{1/2}$ (μM)	k_{cat} (s^{-1})	$k_{cat}/K_{1/2}$ (M^{-1} s^{-1})
ubiquitin	0.41 ± 0.08	2.15 ± 0.34	5.2×10^6
3	170 ± 78	1.89 ± 0.04	1.1×10^4
4	370 ± 50	2.40 ± 0.27	6.5×10^3
5	370 ± 79	0.30 ± 0.13	1.1×10^3
8	469 ± 84	2.90 ± 1.40	6.2×10^3

reverse-phase HPLC to be greater than 95% for all peptides (Figure S1 of the Supporting Information).

PP$_i$−ATP Exchange Assay. The assay was performed as reported previously with minor modification (*21–23*). The E1 enzyme concentrations were obtained from the manufacturer's data sheets and used directly in our calculations. Briefly, incubation reactions were set up with a total volume of 250 μL containing 1 μL of 1.5 μM ubiquitin E1, various amounts of peptide probes, and 50 μL of reaction buffer [500 mM Tris, 25 mM $MgCl_2$, 5 mM ATP, and 1 mM DTT (pH 7.6)]. The mixtures were allowed to equilibrate for 10 min before 1 μL of 0.88 mM PP$_i$ with a specific ^{32}P activity of 25–100 cpm/pmol was added to initiate the reactions. To ensure that only initial exchange velocities within the linear progress curve were measured, time points of 1, 2, 3, 4, and 5 min were chosen; at these points, aliquots of 40 μL of reaction mixtures were withdrawn, and the reactions were quenched by adding 500 μL of a 5% TCA solution. Slurries of 20 μL of activated charcoal in 200 μL of a 5% TCA solution were added to the quenched reaction mixtures, and the mixtures were spun down to remove the supernatants. The charcoal pellets were washed with 600 μL of a 2% TCA solution twice, and activities were measured via a scintillation counter. The velocities for various substrate concentrations were derived, and the $K_{1/2}$ and V_{max} values were subsequently determined by fitting the double-reciprocal plot to the equation $1/v = (K_{1/2}/V_{max})(1/[S]) + 1/V_{max}$. The rate constant, k_{cat}, was calculated using the equation $k_{cat} = V_{max}/[E1]_t$, where $[E1]_t$ is the total enzyme concentration of E1.

E1−Peptide Probe Conjugation Assay. For the screening assay, 10 μL reaction mixtures containing 90 nM ubiquitin E1 and 450 μM peptide probes or 58 nM ubiquitin were incubated in the reaction buffer [50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, and 10 μM ATP (pH 7.6)] at 37 °C for 5 min. For the concentration dependence study, a solution of 90 nM ubiquitin E1 and varying amounts of compound **4** was incubated in 10 μL of reaction buffer at 37 °C for 5 min. The reactions were terminated by adding 2 μL of 6× nonreducing SDS sample buffer, and the mixtures were boiled at 95 °C for 2 min. The biotin-labeled E1 thioesters were resolved from free biotins with an 8 to 16% Tris-Gly SDS−PAGE gel, transferred onto a PVDF membrane, and probed with the VECTASTAIN ABC-AmP detection system following the manufacturer's instructions.

E2−Peptide Probe Conjugation Assay. A solution of 90 nM rabbit E1, 1 μM UbcH7 (E2), and varying amounts of compound **4** in 10 μL of reaction buffer [50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, and 10 μM ATP (pH 7.6)] was incubated at 37 °C for 5 min. Reactions were terminated by adding 2 μL of 6× nonreducing SDS sample buffer, and the mixtures were boiled at 95 °C for 2 min. The biotin-labeled E1 and E2 thioesters were resolved from free biotins with an 8 to 16% Tris-Gly SDS−PAGE gel, transferred onto PVDF membrane, and probed with the VECTASTAIN ABC-AmP detection system following the manufacturer's instructions.

E1-to-E2 Transfer Assay. The assay was performed in a manner similar to that of a previous report (*22*) with some notable modifications. A reaction mixture containing 11 μL of 0.5 μM ubiquitin E1 and 5 μL of 4.5 mM peptide probes in 50 μL of reaction buffer [100 mM Tris, 0.5 mM $MgCl_2$, and 2 mM ATP (pH 7.6)] was incubated at 37 °C for 5 min. Following incubation, 5 μL of 100 mM EDTA was added and the mixture was equilibrated for an additional 5 min at 37 °C. A control sample was then taken before addition of 9 μL of 10 μM UbcH7 to the mixture. At the specified time points, 6 μL aliquots were taken from the incubation mix, and the reaction was quenched by adding 2 μL of 6× nonreducing SDS sample buffer followed by boiling at 95 °C for 5 min. The biotin-labeled E2 thioesters were resolved from free biotin with an 8 to 16% Tris-Gly SDS−PAGE gel, transferred onto a PVDF membrane, and probed with the VECTASTAIN ABC-AmP detection kit following the manufacturer's instructions. The biotin-containing E2 thioester bands were quantified using ImageJ. The time of transfer, $t_{1/2}$, was calculated by fitting the data in SigmaPlot to the equations $[B]_t = [A]_0(1 - e^{-k_{obs}t})$ and $t_{1/2} = 0.6931/k_{obs}$.

Tubulin Conjugation Assay. The tubulin−parkin complex was prepared according to the protocol of Vallee et al. (*24*). Briefly, 3-week-old male Sprague-Dawley rats were decapitated after being anesthetized with halothane (Sigma). Whole brains were collected and homogenized in PEM buffer (0.1 M PIPES, 1 mM EGTA, and 1 mM $MgSO_4$) on ice. After centrifugation at 4 °C first at 30000*g* for 15 min and then at 180000*g* for 90 min, the supernatant was collected, and Taxol and GTP at concentrations of 20 μM and 1 mM, respectively, were added. The solution was warmed to 37 °C to allow tubulin and the associated proteins to reassemble into microtubules. The suspension was then centrifuged through a layer of sucrose, and the pellet was collected and rinsed in PEM buffer at 37 °C. The microtubules and associated proteins, including parkin and the ubiquitination enzymes, were dissociated in PEM buffer at 4 °C to derive the semipurified tubulin−parkin complex which was used directly in our ubiquitination assays. A total of 4 μg of semipurified tubulin−parkin complex and various amounts

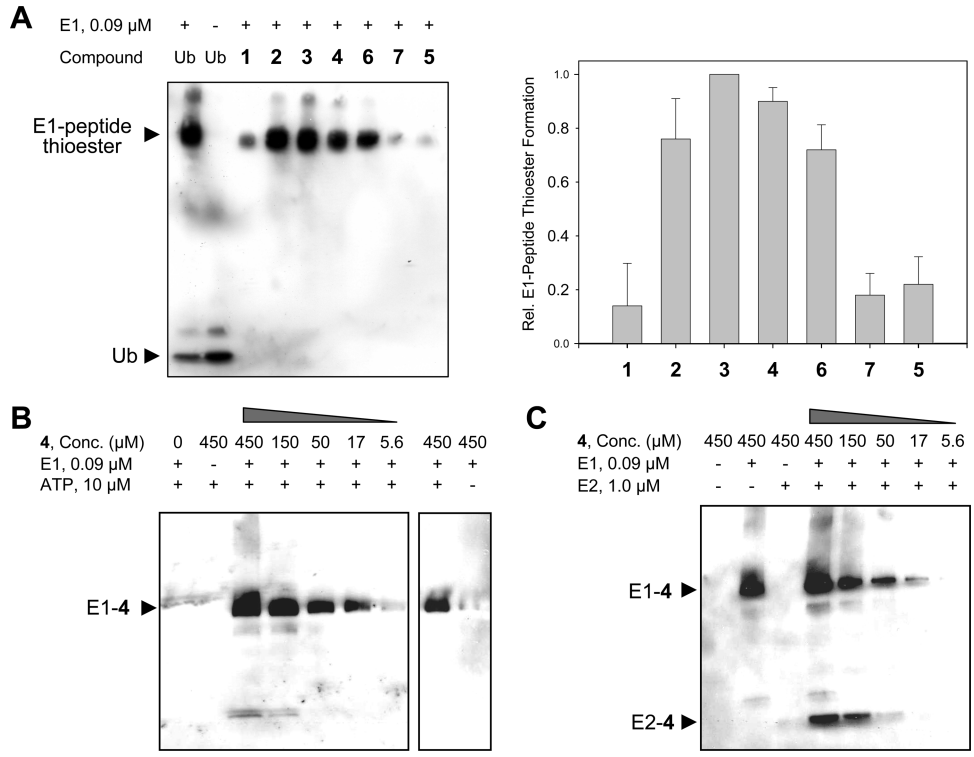


FIGURE 2: Peptide probe activation and transfer monitored by Western blotting. An N-terminally biotinylated ubiquitin was assayed at 58 nM, and all peptide probes were assayed at 450 μM. The attachment of peptide probes to either E1 or E2 enzyme was probed with streptavidin-bound alkaline phosphatase and detected by chemiluminescence. (A) Structure-dependent formation of E1−peptide probe thioesters. The bar graph at right represents the normalized, average E1 thioester formation efficiency of three independent experiments relative to probe **3**. (B) Concentration- and ATP-dependent formation of E1−**4** thioesters. (C) Concentration-dependent formation of E1−**4** and E2−**4** thioesters.

of peptide probes was incubated at 37 °C for 15 min in 10 μL of buffer [50 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, and 10 μM ATP (pH 7.6)]. For the DTT stability assay, 1 μL of 6 M DTT was added (final concentration of 545 mM) and the solution was incubated for an additional 30 min. Reactions were terminated by adding 2 μL of 6× SDS sample buffer containing 100 mM DTT, and the mixtures were boiled at 95 °C for 5 min. The labeled tubulin was resolved from an excess amount of peptide probes with an 8 to 16% Tris-Gly SDS−PAGE gel, transferred onto the PVDF membrane using a semidry protein transfer apparatus, and probed using either the VECTASTAIN ABC-AmP detection system or rabbit polyclonal anti-fluorescein antibodies following the manufacturer's instructions. The intensities of protein bands were quantified using ImageJ.

Fraction II Labeling. Fraction II from rabbit reticulocyte lysates was incubated with ImmunoPure streptavidin beads (Pierce, Rockford, IL) to remove any protein that binds nonspecifically to the detection reagents. An aliquot of cleared fraction II (5.5 μg) was then incubated with various amounts of peptide probes in 10 μL of reaction buffer [100 mM Tris, 3 mM DTT, 5 mM $MgCl_2$, and 2 mM ATP (pH 7.6)] at 37 °C for 10 min. The reactions were terminated by adding 2 μL of 6× SDS sample buffer, and the mixtures were boiled at 95 °C for 5 min. For the screening assay, final concentrations of 450 μM peptide probes were used. The biotin-labeled proteins were resolved from biotin peptide probes with an 8 to 16% Tris-Gly SDS−PAGE gel, transferred onto a PVDF membrane, and blotted with the VECTASTAIN ABC-AmP detection system following the manufacturer's instructions. The labeled proteins were quantified using ImageJ. The apparent substrate constant $K_{m,app}$ was estimated by fitting the data to a hyperbolic two-parameter equation.

RESULTS AND DISCUSSION

Synthesis of Peptide Probes. Given our hypothesis that the carboxyl terminus of ubiquitin is principally responsible for the conjugation activity of ubiquitin, we synthesized a series of peptide probes (**1–8**) by linking either biotin or fluorescein to ubiquitin carboxyl termini of various lengths through a flexible linker (Table 1). The gradual increase in length from **1** to **7** should allow us to probe the minimum recognition sequence required for efficient processing. The replacement of Arg with Ala (compare the recognition sequence of compound **4** to that of **5**) should enable us to probe the activation specificity as LALRGG represents the Nedd8 carboxyl-terminal sequence. All the peptide probes were purified to homogeneity by preparative high-performance liquid chromatography, and their identities were confirmed by electrospray mass spectrometry (Table 1).

Substrate Properties of Peptide Probes. To demonstrate whether these peptide probes can be utilized as substrates by the ubiquitination pathway, we first examined their adenylation by ubiquitin E1 in a PP_i−ATP exchange assay (*21*). For initial screening, the exchange rates were measured at 450 μM for all peptide probes. We found that higher exchange rates were obtained when peptide probes carry either a pentamer sequence (RLRGG) (**3**) or a hexamer sequence (LRLRGG) (**4**). Decreasing (**1** and **2**) or increasing (**6**) chain length or replacing a key Arg residue with Ala (**5**) resulted in lower velocities (Figure S2 of the Supporting Information). Detailed kinetic analysis was then carried out

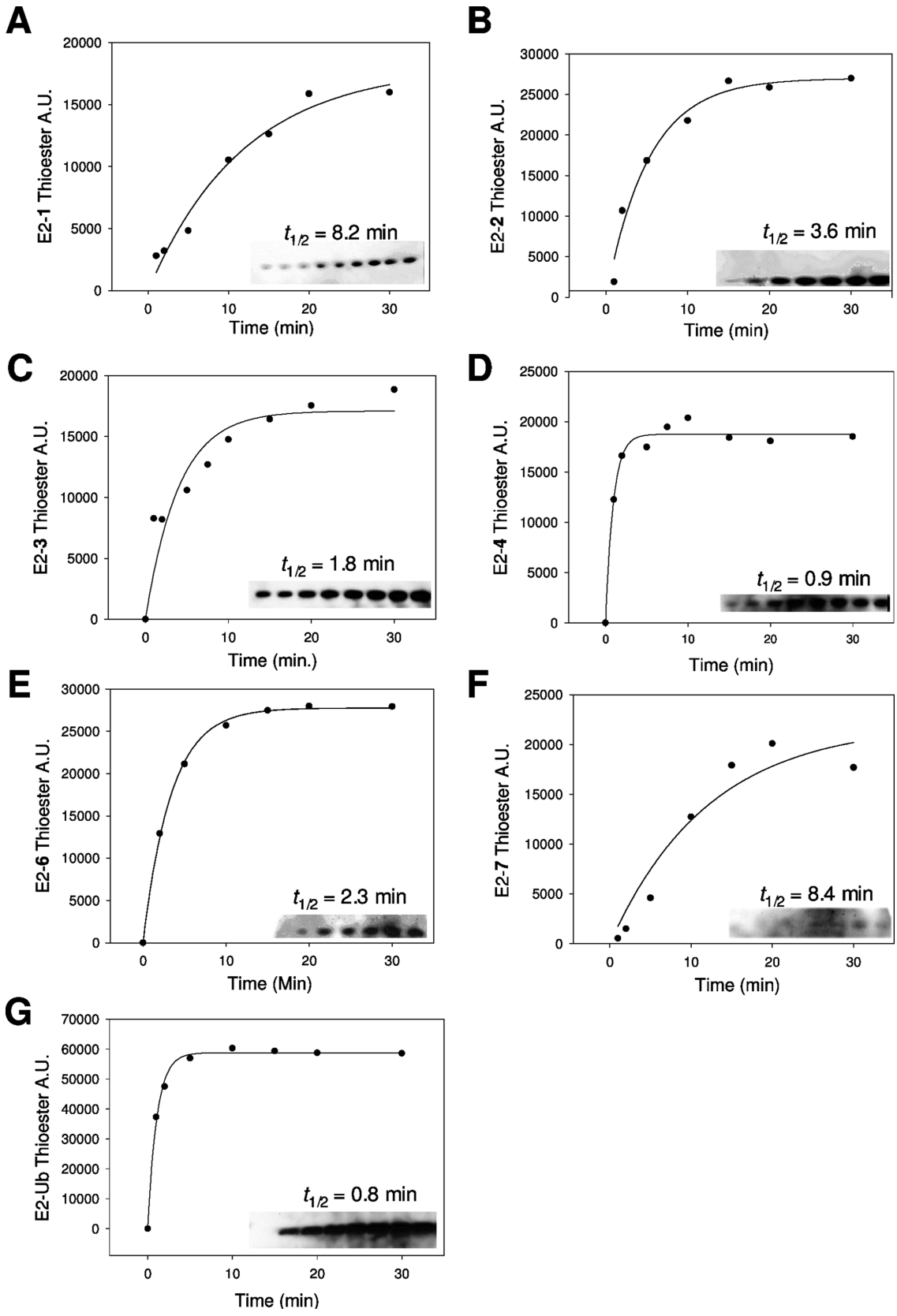


FIGURE 3: Time courses of probe transfer from the E1−thioester form to E2 under steady-state conditions. The formation of E2−probe thioesters was monitored by following the incorporation of biotin into the E2 enzymes in Western blots, and they are shown as insets in the graphs. The thioester adducts were probed with streptavidin-bound alkaline phosphatase and detected by chemiluminescence. The $t_{1/2}$ values were derived by fitting the curve to the equations $[B]_t = [A]_0(1 - e^{-k_{obs}t})$ and $t_{1/2} = 0.6931/k_{obs}$. The values shown were averaged over two independent experiments: (A) probe **1**, (B) probe **2**, (C) probe **3**, (D) probe **4**, (E) probe **6**, (F) probe **7**, and (G) biotin-Ub.

for the active compounds, and the data are collected in Table 2. Notably, compound **3** exhibited the highest activity among all the peptide probes with the second-order kinetic constant ($k_{cat}/K_{1/2}$) determined to be 1.1×10^4 M^{-1} s^{-1}. By comparison, $k_{cat}/K_{1/2}$ for ubiquitin was determined to be 5.2×10^6 M^{-1} s^{-1} (comparable to the literature value, 3.5×10^6 M^{-1}

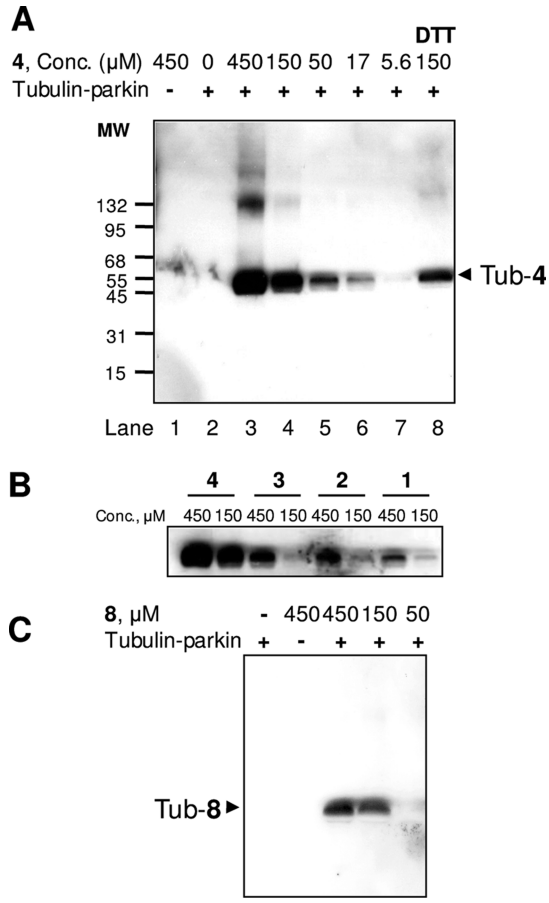


FIGURE 4: Western blot analysis of tubulin labeling by peptide probes in the tubulin−parkin complex. The biotin labeling of tubulins was probed with streptavidin-bound alkaline phosphatase and detected by chemiluminescence; the fluorescein labeling was probed with an anti-fluorescein antibody and detected by chemiluminescence. (A) Concentration-dependent labeling of tubulin by probe **4**. (B) Structure-dependent labeling of tubulin by peptide probes **1**−**4**. (C) Labeling of tubulin by fluorescent probe **8**.

s^{-1}) (*25*), which is roughly 470-fold higher than that of peptide probe **3**. The generally lower activities for peptide probes can be attributed primarily to a significant reduction in $K_{1/2}$ (Table 2), which could be the result of smaller surface areas in the peptide probes as compared to the full-length ubiquitin. Since $K_{1/2}$ is a complex constant composed of internal rate and equilibrium constants for the formation of a ternary complex, $E1^{AMP\text{-}3:PP_i}S$-**3**, the roughly 400-fold drop in $K_{1/2}$ for compound **3** relative to that of ubiquitin indicates a less robust ternary complex leading to ATP formation. Compounds **4** and **8** carrying the recognition sequence LRLRGG exhibited nearly identical, second-highest activity, presumably due to less optimum ternary complexes compared to compound **3**. In addition, compound **5** exhibited an approximate 6-fold reduction in activity compared to that of compound **4**, in agreement with previous reports that Arg^{72} is a specificity-determining residue for ubiquitin in its binding to the E1 adenylation site and vital for its adenylation activity (*25, 26*). Compared to the reported peptide substrates (*16*), the biotin- and fluorescein-modified peptide probes (**1–8**) exhibited much higher activities in the PP_i−ATP exchange assay, presumably due to the positive effect of the biotin or fluorescein moiety in providing additional hydrophobic surface for the binding.

To examine whether the peptide adenylates can be internally trapped by the active site Cys^{632} in ubiquitin E1, the formation of E1−peptide probe thioesters was measured in an end point assay. Figure 2A shows formation of a sequence-dependent E1 thioester for the biotinylated peptide probes with compounds **3** and **4** being the most efficient substrates, consistent with our PP_i−ATP exchange assay result (Table 2). Incubation of various amounts of compound **4** with E1 for 5 min led to concentration-dependent formation of E1−**4** thioester (Figure 2B). The E1−**4** adduct was labile to the DTT treatment, consistent with the formation of a thioester bond (Figure S3 of the Supporting Information). In addition, the biotin adducts were not observed when ATP was excluded from the reaction buffer (Figure 2B), indicating the E1−peptide probe conjugation reaction is indeed ATP-dependent. To examine whether the activated E1−peptide probe thioesters can be transferred to E2, we incubated peptide probe **4** at various concentrations with E1 and UbcH7 (E2) in the presence of 10 μM ATP. Concentration-dependent formation of E1−**4** and E2−**4** thioesters was observed when the concentrations were higher than 50 μM (Figure 2C). It is noteworthy that the incubation of probe **4** with E2 in the absence of E1 did not give rise to the E2−**4** thioester (lane 3 in Figure 2C), indicating the peptide transfer is mediated through the E1−**4** thioester intermediate. Both E1−**4** and E2−**4** intermediates were labile to the DTT treatment, confirming that their linkages are mediated through thioester bonds (Figure S4 of the Supporting Information). By comparison, incubation of E1 and E2 with ubiquitin yielded a similar trend with the thioesters appearing at concentrations as low as 64 nM (Figure S5 of the Supporting Information).

To examine the rate of peptide probe transfer from E1 to E2, we carried out a steady-state time course experiment in which the extent of the formation of E2−peptide probe thioester was monitored over 30 min while the concentrations of E1−peptide probe thioesters remained constant (Figure 3). The time of transfer, $t_{1/2}$, showed a strong structural dependence, with the hexamer sequence (compound **4**) yielding the fastest $t_{1/2}$, 0.9 min (Figure 3D). Either extending (e.g., **7**, Figure 3F) or shortening (e.g., **1**, Figure 3A) the recognition sequence led to a significant attenuation of $t_{1/2}$. By comparison, full-length ubiquitin exhibited a $t_{1/2}$ of 0.8 min (Figure 3G), essentially identical to that of compound **4**. It has been shown that the E1-bound ubiquitin is important for an efficient E2−E1 interaction as free E2 binds tightly to the ubiquitin-loaded E1 with a subnanomolar K_d (*27*) but weakly to the free E1 (*4*). Our transfer studies with the ubiquitin carboxyl-terminally derived peptide probes corroborate these earlier observations as the transfer efficiency ($t_{1/2}$) was evidently dependent on structures of the E1−peptide probe thioesters. It appears a hexameric sequence is sufficient for an ordered thioester transfer as $t_{1/2}$ for compound **4** is essentially identical to that of full-length ubiquitin. The lower transfer rates observed for peptide probes with either shorter (**1**, **2**, and **3**) or longer recognition sequences (**6** and **7**) suggest that perhaps a certain conformational preorganization of E1−peptide probe thioesters might be necessary in interacting effectively with E2.

Parkin-Mediated Labeling of Tubulin by Peptide Probes. Tubulins are basic building blocks of microtubules, an important component of the eukaryotic cytoskeleton contributing to cell shape and movement (*28*). Tubulins employ

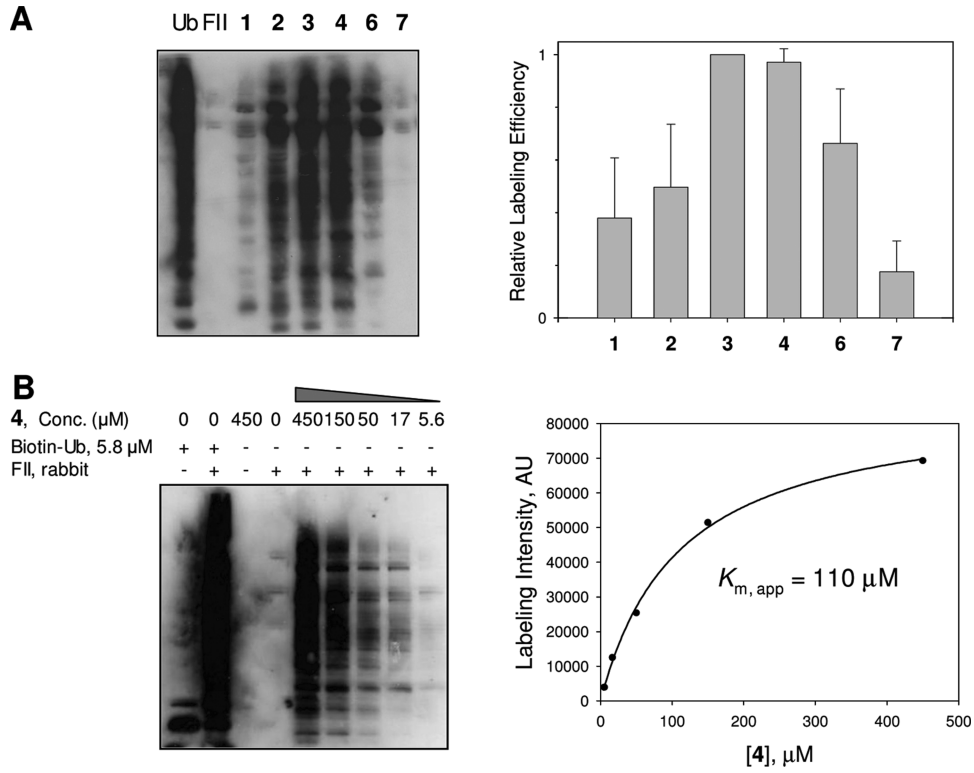


FIGURE 5: Western blot analysis of reticulocyte fraction II labeling by peptide probes. The labeling was probed with streptavidin-bound alkaline phosphatase and detected by chemiluminescence. (A) Structure-dependent labeling of fraction II by peptide probes. Peptide probes were assayed at 450 μM, while ubiquitin was assayed at 0.58 μM. The labeling efficiency was quantified by integrating the density across individual lanes. The bar graph on the right represents the normalized, average labeling efficiency of three independent experiments relative to probe **3**. (B) Concentration-dependent labeling of fraction II by probe **4**. The overall intensity of each lane was quantified and plotted against the concentrations of probe **4** in the graph, which upon curve fitting gave an apparent substrate constant $K_{m,app}$ of 110 μM.

an array of posttranslational modifications, including acetylation, tyrosination, and polyglutamylation, to further modulate the binding of microtubule-associated proteins (MAPs) to microtubules (*29*). We have recently shown that parkin, an E3 ligase implicated in Parkinson's disease, binds tightly to microtubules and catalyzes tubulin ubiquitination and subsequent degradation in the cultured cells (*30*).

To investigate whether these peptide probes can be utilized in the parkin-mediated labeling of tubulins, we incubated peptide probe **4** with a semipurified rat brain tubulin−parkin complex, which contains E1 and E2 activities, in a reaction buffer supplemented with 10 μM ATP. Concentration-dependent biotinylation of a 55 kDa protein was observed in the concentration range of 17−450 μM (Figure 4A). The identity of the labeled bands was confirmed to be tubulin both by immunoblotting with an anti-α-tubulin antibody and by their abilities to reassemble into high-molecular mass tubulin oligomers after a prolonged incubation (upper band in lane 3, Figure 4A) which was completely abolished in the presence of 10 μM colchicine, a microtubule assembly inhibitor (Figure S6 of the Supporting Information). The labeling of tubulin appears to be mediated via isopeptide linkages as the biotinylated adduct was stable to the 150 mM DTT treatment (lane 8, Figure 4A). However, preincubation of the tubulin−parkin complex with either 20 mM mercaptoethanol or 600 mM DTT completely abolished the labeling, consistent with the requirement of a nonreducing buffer condition during formation of the thioester (Figure S7 of the Supporting Information). The extent of biotinylation was estimated to be 8% after incubation for 30 min with 450 μM peptide probe **4** using biotin-Ub as an internal control (Figure S8 of the Supporting Information). In addition, compound **4** exhibited the highest activity in labeling tubulins among the peptide probes that were tested (Figure 4B), which is consistent with its higher activity in the adenylation assay as well as the E1-to-E2 transthiolation assay. Importantly, in a control experiment, incubating the tubulin−parkin complex with a biotin analogue lacking the recognition sequence, biotinyl aminohexanoic acid, did not lead to formation of biotin adducts (Figure S7 of the Supporting Information). To explore whether peptide probes carrying a fluorescent probe, such as **8**, can be used selectively by the ubiquitination pathway in the parkin-mediated labeling of tubulins, compound **8** was incubated with the tubulin−parkin complex. Indeed, the formation of fluorescein-labeled tubulin was detected using an anti-fluorescein antibody when the concentrations of probe **8** were greater than 150 μM (Figure 4C). However, direct monitoring of the fluorescent bands by a PhosphoImager was not successful, presumably due to a low labeling efficiency.

Labeling of Fraction II Proteins by Peptide Probes. To demonstrate that these peptide probes can be used to probe ubiquitination targets in cellular extracts, we incubated these compounds with a ubiquitin-free reticulocyte fraction II cell extract in which ubiquitin E1, various E2, and hundreds of E3 ligase activities were present (*31, 32*). A structure-dependent labeling of fraction II proteins by peptide probes was again observed (Figure 5A), which bears a remarkable resemblance to the trend seen in both the E1 conjugation assay (Figure 2A) and the E1-to-E2 transthiolation assay (Figure 3). These conjugates are not E1− and E2−peptide probe thioesters as treatment with 100 mM DTT after

incubation did not lead to the reduction of their intensities on the SDS−PAGE gel (Figure S9 of the Supporting Information). Since overall labeling efficiency reflects combined effects of three successive steps in the ubiquitination pathway, the high level of correlation between labeling efficiency and substrate properties suggests the extent of labeling is largely determined by the absolute amount of activated E2−peptide probe thioesters. In addition, the similar labeling pattern (the number of bands formed and their relative intensity) seen in Figure 5A suggests that target specificity is exclusively achieved by the E3 ligases present in the reticulocyte lysate. However, much higher concentrations of peptide probes were needed to produce the same level of target labeling relative to the full-length ubiquitin (450 μM vs 0.58 μM), which can be attributed to the significantly lower activation efficiency for peptide probes by E1. Furthermore, a hyperbolic concentration-dependent labeling of fraction II by probe **4** was observed with an apparent substrate constant $K_{m,app}$ of 110 μM (Figure 5B).

In summary, we have demonstrated that peptide probes for protein ubiquitination can be designed by attaching biophysical probes to the ubiquitin C-terminal recognition sequences. These peptide probes were employed to label a semipurified ubiquitination substrate, tubulin, as well as many proteins in fraction II of the rabbit reticulocyte extract by functioning as modest, yet sufficient, substrates for the ubiquitination pathway. Their substrate properties were further corroborated by their structure-dependent activities in the E1-catalyzed adenylation, E1−peptide probe thioester formation, and E1-to-E2 transthiolation. While the substrate properties of these UBT-derived peptide probes are modest relative to that of the native ubiquitin, it is reasonable to expect that they can be further improved through structural optimization on the basis of the structure–activity relationship data generated from this study.

Since there are reportedly 600 E3 ligases in the human genome that selectively recruit intracellular proteins for the proteasome-dependent proteolysis (*33*), it is imperative that targeting specificity of each E3 ligase can be experimentally established. To this end, one successful strategy was to overexpress an epitope-tagged ubiquitin, e.g., FLAG-Ub, to modify a select subset of substrates upon specific E3 induction and then isolate and characterize the tagged protein targets (*10, 34*). However, the dynamic range and quantitative power of this approach might be limited because ubiquitin overexpression invariably leads to an increased, yet undesirable, level of target degradation. In this regard, despite their generally lower activities, these peptide probes may provide valuable proteomic tools because (i) the substrate−peptide adducts are likely resistant to the proteasome-dependent proteolysis due to the disruption of polyubiquitin chain growth (*35–37*) and (ii) the isopeptide bonds between the protein substrates (or ubiquitin in the case of ubiquitin chain growth) and peptide probes are likely less susceptible to the ubiquitin C-terminal hydrolase (UCH)-mediated hydrolysis (*38*). Furthermore, since protein ubiquitination specificity is controlled primarily through temporospatial expression of unique E3 ligases in response to specific ubiquitination signals (*6*), it might be possible to harness this endogenous biochemical machinery to label specific proteins (ubiquitination substrates) with peptide probes in living cells through overexpression of the cognate E3 ligases. Compared to other enzyme-mediated labeling of proteins with small molecules (*20, 39–42*), this ubiquitination pathway-based approach does not require the fusion of targeting domains (or short peptide sequences) to the protein of interest and, in principle, can be applied to native proteins in their endogenous environments. The efforts to optimize the substrate properties of peptide probes by incorporating organic scaffolds and to examine the proteolytic stability of the protein−peptide probe adducts against proteasomes as well as UCH are currently underway.

SUPPORTING INFORMATION AVAILABLE

HPLC traces of peptide probes **1**−**8** (Figure S1), time courses of peptide probe adenylation reactions measured by the [^{32}P]PP$_i$−ATP exchange assay (Figure S2), E1−**4** thioesters labile to the DTT treatment (Figure S3), E1−**4** and E2−**4** adducts labile to the DTT treatment (Figure S4), ubiquitin activation and transfer catalyzed by E1 and E2 (Figure S5), colchicine inhibiting the formation of high-molecular mass oligomers of the probe-labeled tubulins (Figure S6), labeling of tubulin by peptide probe **4** (Figure S7), time course of tubulin labeling by peptide probe **4** over 30 min (Figure S8), and structure-dependent labeling of fraction II by peptide probes (Figure S9). This material is available free of charge via the Internet at http://pubs.acs.org.

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