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Using Glycinylation, a Chemical Derivatization Technique, for the Quantitation of Ubiquitinated Proteins

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Abstract

The quantitation of lysine post-translational modifications (PTMs) by bottom-up mass spectrometry is convoluted by the need for analogous derivatives and the production of different tryptic peptides from the unmodified and modified versions of a protein. Chemical derivatization of lysines prior to enzymatic digestion circumvents these problems and has proven to be a successful method for lysine PTM quantitation. The most notable example is the use of deuterioacetylation to quantitate lysine acetylation. In this work, levels of lysine ubiquitination were quantitated using a structurally homologous label that is chemically similar to the di-glycine (GlyGly)-tag, which is left at the ubiquitination site upon trypsinolysis. The LC-MS analysis of a chemically equivalent mono-glycine (Gly)-tag that is analogous to the corresponding GlyGly-tag proved that the mono-glycine tag can be used for the quantitation of ubiquitination. A glycinylation protocol was then established for the derivatization of proteins to label unmodified lysine residues with a single glycine tag. Ubiquitin multimers were used to show that after glycinylation and tryptic digestion, the mass spectrometric response from the corresponding analogous tagged peptides could be compared for relative quantitation. For a proof of principle regarding the applicability of this technique to the analysis of ubiquitination in biological samples, the glycinylation technique was used to quantitate the increase in mono-ubiquitinated histone H2B that is observed in yeast which lack the enzyme responsible for deubiquitinating H2B-K123, compared to wild-type yeast.

Introduction

The ATP-dependent, post-translational attachment of ubiquitin to proteins, called ubiquitination, is a well-known regulation and signaling tool used by the cell to control an array of cellular processes such as endocytosis, cell cycle progression, protein sorting and inflammation.¹ Ubiquitination involves the formation of an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue in the substrate protein.² The modification of proteins with ubiquitin can occur as either mono-ubiquitination, multi-ubiquitination (multiple mono-ubiquitination events), or poly-ubiquitination.³ Poly-ubiquitin chains involve the successive conjugation of ubiquitin moieties onto previously attached ubiquitins using any of the seven lysine residues of ubiquitin (K₆, K₁₁, K₂₇, K₂₉, K₃₃, K₄₈, or K₆₃); of which the most commonly used are K₄₈ and K₆₃.¹ Poly-ubiquitin chains can have diverse topologies with either homogeneous or

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This paper is dedicated to the memory of the senior author (R.J. Cotter).

Supporting Information

Detailed experimental methods and additional information as noted in the main text.

heterogeneous linkages and can contain forks, when one ubiquitin unit in the chain is multi-ubiquitinated.⁴ Numerous proteins in the ubiquitin network contain ubiquitin-binding domains (UBDs) that read and transmit the ubiquitin signal to instigate a distinct cellular outcome.⁵ For example, K₄₈-linked poly-ubiquitin chains lead to proteasomal degradation, whereas K₆₃-linked poly-ubiquitination has been linked to kinase activation, DNA repair, and ribosome function. Mono-ubiquitination usually determines protein localization, transcriptional activation, and chromatin structure.³ Like most post-translational modifications (PTMs), ubiquitination is a reversible modification; this action is carried out by deubiquitinating enzymes (DUBs).⁵

Bottom-up mass spectrometry has been successful in characterizing ubiquitinated proteins since upon trypsin digestion, the two C-terminal glycine residues of ubiquitin remain on the modified lysine of the substrate peptide.¹ This di-peptide remnant serves as a mass tag (114.04 Da) for the unambiguous localization of the ubiquitination site when using tandem mass spectrometry.⁵ The tandem mass spectrum also contains sequence information from the substrate peptide that can be used to identify the ubiquitinated protein. Shotgun proteomics is routinely used for the identification of unknown ubiquitinated proteins and the characterization of ubiquitination sites.⁵ An N-terminal sulfonation technique is also available for the targeted detection and straightforward *de novo* sequencing of ubiquitinated peptides.^{6, 7} However, the detection of ubiquitination is plagued by low stoichiometric levels of the modified form of the protein and quick turnover of ubiquitinated proteins by proteasome-dependent proteolysis.^{4, 5} Enrichment strategies are frequently used to increase the amount of ubiquitinated substrate in the sample. Examples include the use of epitope-tagged ubiquitin or substrate proteins (i.e. His, FLAG, HA, biotin or Myc), ubiquitin specific antibodies, anti-K-ε-GG remnant antibodies⁸, or ubiquitin-binding proteins.^{4, 9, 10}

Current efforts in the ubiquitin field have focused on using quantitative mass spectrometric experiments to compare how the ubiquitinome changes under different physiological conditions; for example, upon proteasomal inhibition^{11, 12} or after genetic deletion of certain E2s, E3s or DUBs.¹ However, traditional trypsin-based bottom-up methods cannot be used to quantitate lysine modifications as compared to unmodified lysine residues because different tryptic peptides will be produced from the modified and unmodified forms of the protein (since trypsin cleaves at unmodified lysines). Instead, chemical derivatization must be performed before enzymatic digestion to block unmodified lysine residues; then trypsin will only cleave at arginines, producing unmodified and modified peptides of the same length.¹³ This “mock Arg-C” digestion also produces longer peptides to provide a pseudo middle-down approach, resulting in greater “lysine coverage” and the ability to correlate modifications on multiple lysines within the same peptide, such as multi-ubiquitinated substrates or forked poly-ubiquitin chains.^{9, 14}

Lysine derivatization schemes that allow for the relative quantitation of PTMs generally involve the incorporation of stable isotope or structural analogs of the endogenous modification. For example, the quantitation of lysine acetylation can be carried out using deuterioacetylation^{15, 16} or propionylation derivatization.¹⁷ The absolute quantification (AQUA) method, on the other hand, is an elegant way to determine the exact amount of a protein or PTM. This method involves the synthesis of an isotope-labeled reference peptide with the same sequence as a tryptic peptide from the protein of interest.¹⁸ AQUA has been successfully used to characterize the length and topology of poly-ubiquitin chains, since the sequence of each lysine-containing ubiquitin peptide is known.^{19, 20} However, because the AQUA strategy requires the identification of the substrate and the localization of the ubiquitination site for the synthesis of the isotope-labeled internal standards, the technique is restricted to known analytes and is incompatible with discovery-based platforms.¹⁸

In this work, a derivatization method was developed for the quantitation of lysine ubiquitination. A structurally analogous derivative, having chemical similarity to the GlyGly-tag, was used for the quantitative comparison between ubiquitinated and non-ubiquitinated proteins. With this technique, the identification and characterization of unknown ubiquitination sites can be performed in parallel with their quantitation. The technique was optimized using recombinant mono- and poly-ubiquitin and was demonstrated to be applicable to biological samples based on the analysis of histone H2B isolated from yeast.

Experimental

Synthetic Branched Peptides

The ubiquitin peptide, $_{43}\text{LIFAGK}_{48}\text{QLEDGR}_{54}$, was synthesized with either a Gly-tag or a GlyGly-tag on the K_{48} residue by Jodie Franklin of the JHMI Synthesis and Sequencing Facility. The peptides were combined in solution at a concentration of $1\text{ pmol}/\mu\text{L}$ of each peptide in 0.1% formic acid and $2\mu\text{L}$ of the solution was subjected to quantitative LC-MS/MS analysis.

K_{48} -linked Ubiquitin

Ubiquitin monomer and K_{48} -linked ubiquitin polymers (di-, tri-, tetra-, and penta-ubiquitin) were purchased from Boston Biochem (Cambridge, MA). Stock solutions of each were prepared in water and the proteins were combined according to Table 1 to establish a range of percent of ubiquitinated Ub- K_{48} . All samples were dried in a SpeedVac before derivatization.

Glycinylation Reaction

The protein sample was resuspended in $20\mu\text{L}$ water with $2\mu\text{L}$ of ammonium hydroxide to maintain a pH of approximately 8.5 during the derivatization reaction. Boc-glycine anhydride was synthesized (David J. Meyers of the JHU SOM Synthetic Core Facility) by coupling Boc-glycine with N,N' -dicyclohexylcarbodiimide (DCC) in methylene chloride.²¹ Boc-glycine anhydride was dissolved in isopropyl alcohol at a concentration of $75\text{ nmol}/\mu\text{L}$ and then $50\mu\text{L}$ ($3.75\mu\text{mol}$) was added to the sample. The derivatization reaction was performed for 30 min at 55°C . The samples were dried in a SpeedVac. After trypsin digestion, the boc protecting groups were removed with $50\mu\text{L}$ neat TFA at room temperature for 1 hour. The sample was washed with 1 mL cold diethyl ether and then dried in a SpeedVac. Cold ether washes were performed two additional times before mass spectrometric analysis.

Trypsin Digestion

Samples were resuspended in $99\mu\text{L}$ 100 mM ammonium bicarbonate to which $0.2\mu\text{g}$ of trypsin in $1\mu\text{L}$ of 1 mM HCl was added. Trypsin digestion was performed overnight at 37°C and then quenched with $1\mu\text{L}$ formic acid (FA) before the sample was dried in a SpeedVac.

Histone H2B from Yeast

Two yeast strains were obtained from the KanMX knockout library (courtesy of Dr. Jef Boeke): a wild-type *Saccharomyces cerevisiae* strain, BY4741 (genotype - MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0), and a ΔUbp8 strain. The plasmid (pJD106 FLAG-His10-HTB1 LEU CEN) was transformed into both strains as a source of His-tagged histone H2B. One liter of each yeast strain was grown to an approximate OD of 1 in -leu media from which

histone H2B was purified as previously described.²² Further experimental details can be found in the supporting information.

Western Blot

Two aliquots of 30 μ L from both eluates were each diluted with 10 μ L LDS sample buffer and separated on 1.0 mm thick NuPAGE 4–12% Bis-Tris gels (Invitrogen) using MES SDS running buffer. An anti-FLAG Western blot was performed on the first gel and an anti-ubiquitin Western blot was performed on the second gel. For the anti-FLAG Western blot, the mouse monoclonal anti-FLAG M2 antibody (Sigma F1804) was used as the primary antibody at a dilution of 1:1000. For the anti-Ub Western blot, the mouse monoclonal anti-ubiquitin antibody (Santa Cruz P4D1) was used as the primary antibody at a dilution of 1:1000. Further experimental details can be found in the supporting information.

LC-MS/MS Analyses

Tryptic peptides were resuspended in 0.1% FA and separated on a reversed phase C18 column using an acetonitrile gradient. Mass spectrometric analyses were performed on either a Shimadzu LCMS-IT-TOF (using microflow LC conditions at 0.3 mL/min) or a Thermo LTQ-OrbitrapXL (using nanoflow LC conditions at 300 nL/min). Quantitative methods involved full MS scans only with narrowed mass ranges around each analyte of interest. Quantitation was based on the automatic peak integration of extracted ion chromatograms using instrument control software (LCMSsolution for the LCMS-IT-TOF data and Xcalibur for the LTQ-OrbitrapXL data). Data-dependent methods were used to acquire MS/MS spectra. Further experimental details can be found in the supporting information.

Results and Discussion

A single glycine label was used as a chemically similar derivative tag to the GlyGly moiety. In order to test the equivalency of the mono-glycine tag to the di-glycine tag, the K₄₈-containing peptide from ubiquitin was synthesized with both labels (₄₃LIFAGK₄₈(G)QLEDGR₅₄ and ₄₃LIFAGK₄₈(GG)QLEDGR₅₄). To serve as a true structural homologue, with respect to LC-MS/MS analysis, the derivative-tagged peptide would need to have the same retention time, charge state, and ionization efficiency as the GlyGly-tagged peptide. Therefore the two peptides were combined in equimolar amounts, to make a sample that was 50% ubiquitinated (Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-G)), and then subjected to LC-MS/MS analysis. Figure 1(A) shows the extracted ion chromatograms (XICs) for the +2 and the +3 charge states of the Ub-K₄₈-GG and Ub-K₄₈-G peptides and Figure 1(B) shows the averaged MS1 spectrum for the +2 charge state of the two peptides. Both of the tagged Ub-K₄₈ peptides had the same retention times and charge states. The peaks in each XIC were integrated and the total peak area for both charge states of each peptide was used to determine a percent of ubiquitinated protein, Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-G), of 50.4 $\pm$ 1.4%.²³ This shows that the two peptides also have similar ionization efficiencies and proves that the Gly-tag can be used as an adequate derivative to the GlyGly-tag for the quantitation of lysine ubiquitination via mass spectrometry.

A protocol for the glycylation of proteins to chemically add a single glycine tag to unmodified lysine residues, using boc-glycine anhydride, was then developed (Figure 2). Reaction conditions for the glycylation protocol were based on an acetylation protocol that was previously optimized in the lab^{24–27} and was further optimized to force the derivatization to completion and limit the presence of unmodified lysines (Figure S1). The most successful protocol involved resuspension of the sample in water, the addition of a small volume of ammonium hydroxide, followed by the addition of 75 nmol/ μ L

derivatization reagent in isopropanol. For effective derivatization to occur, it is important that the pH of the reaction be maintained at ~8 since the anhydride will undergo spontaneous hydrolysis in solution and the subsequent production of acid will reduce the pH of the solution.²⁸ The glycine anhydride reagent required boc-protecting groups to prevent the reagent from cyclizing upon reaction with itself. The reaction was then performed for 30 min at 55°C to derivatize unmodified lysine residues with a boc-glycine moiety. After trypsin digestion, the peptides were deprotected with TFA to remove the boc group. Since TFA is known to cause signal suppression during mass spectrometry with ESI²⁹, the amount of TFA in the sample was reduced by multiple washes with cold diethyl ether. This glycylation protocol results in the tagging of the non-ubiquitinated substrate with a mono-glycine tag while the naturally ubiquitinated substrate peptide contains a di-glycine tag. The mass spectrometric signal of the corresponding tryptic peptides that contain the ubiquitination site can be compared to determine the relative ratio of ubiquitinated protein present in the sample compared to non-ubiquitinated protein. If a neighboring lysine on the same peptide is partially occupied by a different PTM, the glycylation technique will not be applicable since the method will produce different peptides; either with the other modification when the endogenous PTM is present or with the mono-glycine modification when the endogenous PTM is not present.

Di-ubiquitin was an ideal substrate for the evaluation of the glycylation reaction since it represents a sample that is 50% ubiquitinated. Figure 3(A) shows the XICs for the +2 and the +3 charge states of the Ub-K₄₈-GG and Ub-K₄₈-G peptides and Figure 3(B) shows the averaged MS1 spectrum of the +2 charge state of the two peptides for glycylation K₄₈-linked di-ubiquitin. Again, it is observed that the two tagged peptides have nearly identical retention times and the same charge states. The derivatization and trypsin digestion of di-ubiquitin should in theory produce the two tagged peptides in an equimolar ratio. However, the total peak area from each tagged peptide was calculated and the average Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-G) observed for glycylation di-ubiquitin was 62.0±1.7%. This percent of ubiquitinated protein to total protein is higher than the 50.4% observed for the synthetic peptides and can be attributed to the tertiary structure of the protein, which results in the inaccessibility of the derivatization reaction or tryptic digestion to di-ubiquitin. Consequently, the glycylation technique will be unique to each proteomic experiment since the ratios of the two tagged peptides will depend on the structure of the protein being studied and the environment of the reaction. Therefore, this technique can only be used to determine relative changes of ubiquitination occurring at the same site and on the same protein between different samples. It cannot be used as an absolute quantitation method to determine the amount of ubiquitination within a single sample unless a calibration curve for the protein of interest can be established or external standardization is performed.

Figure S2 (A) is an MS/MS spectrum of the GlyGly-tagged Ub-K₄₈ peptide from the glycylation di-ubiquitin. Both the *b*- and *y*-ion series confirm the presence of a di-glycine tag on the K₄₈ residue based on an increase in mass of 114.04 Da on an unmodified lysine residue (128.09 Da). Figure S2 (B) is an MS/MS spectrum of the Gly-tagged Ub-K₄₈ peptide from the same derivatized sample. The mass difference between *b*₅-*b*₆ and *y*₆-*y*₇ corresponds to a lysine residue with one glycine moiety (57.02 Da) added. This data demonstrates that unmodified lysine residues were successfully derivatized with a mono-glycine tag.

A universal relationship over a range of percent of ubiquitination needed to be established between the two tagged peptides to ascertain whether the glycylation technique can be used for quantitation.³⁰ To accomplish this, mixtures of di-ubiquitin and ubiquitin monomers as well as tri-, tetra-, and penta-ubiquitin alone were prepared, as described in Table 1, to provide percent of ubiquitinated Ub-K₄₈ ranging from 80% to 17%. Figure S3 is

the calibration curve observed for K₄₈-linked ubiquitin using the glycinylation technique. The experimental relationship established between the two tagged peptides was linear and reproducible throughout the entire data set with a trendline equation of $y=0.6921x+27.489$ and an $R^2=0.995$.

To demonstrate the utility of this technique, glycinylation was used to quantitate the increase in ubiquitinated histone H2B that is known to occur when the deubiquitinating enzyme (DUB), Ubp8, is deleted from yeast cells.³¹ Histones are small, basic proteins that organize DNA into chromosomes. Histones are not, however, inert structural elements; they regulate gene expression through the PTMs that occur on their N- and C-terminal tails.³² The ubiquitin-conjugating enzyme (Ubc), Rad6, is responsible for the ubiquitination of K₁₂₃ on the C-terminal tail of histone H2B and this modification has been implicated in the control of mitotic cell growth and meiosis.³³ It has also been estimated that approximately 10% of total cellular histone H2B is ubiquitinated in wild-type yeast.³³ In the research by Henry *et al.*, which established that Ubp8 is responsible for the deubiquitination of H2B-K₁₂₃, Western blots from wild-type yeast were compared to Δ Ubp8 and Δ Rad6 strains. The slight levels of ubiquitinated H2B that were observed in the wild-type yeast increased in the Δ Ubp8 yeast and were eliminated altogether in the Δ Rad6 yeast.³¹ However, using gel spot intensities to accurately quantitate the observed changes in ubiquitinated histone H2B is difficult and usually unreliable.³⁴ This is mainly due to the bias of gels for higher abundant species and the inability to discriminate between overlaying spots.¹⁸ In addition, antibodies that are used for detection are not always selective, as they are known to pose problems with cross-reactivity and epitope occlusion.³⁵ Instead, we demonstrate how the glycinylation technique can be used to quantitate the level of histone H2B ubiquitination.

FLAG- and His-tagged histone H2B was expressed in wild-type (WT) and Δ Ubp8 yeast strains and then isolated with a His-tag pull down to isolate both the unmodified and modified versions of histone H2B. Enrichment of the modified form or gel separation methods cannot be used with this technique because the unmodified version of the protein is used in the quantitation. Figure 4(A) is an α -FLAG Western blot that shows both the unmodified and the ubiquitinated forms of histone H2B and Figure 4(B) is an α -ubiquitin Western blot that only shows the ubiquitinated form of histone H2B, from both strains. By a visual comparison of the wild-type Ub-H2B band to the Δ Ubp8 Ub-H2B band, it is observed that the levels of ubiquitinated histone H2B increase upon deletion of Ubp8.

The glycinylated histone peptides were analyzed on a LTQ-OrbitrapXL mass spectrometer coupled to a nanoLC. To prove that changing instrument platforms would not affect the quantitation results, equimolar amounts of the synthetic Ub-K₄₈ peptides were analyzed again. The Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-G) was found to be the same as it was on the LCMS-IT-TOF ($51.8\pm0.8\%$, data not shown). Figure S4 shows the averaged mass spectrum from the wild-type yeast to illustrate the low levels of endogenous ubiquitinated histone H2B, represented by the H2B-K₁₂₃-GG peak, compared to the much higher levels of unmodified histone H2B, represented by the H2B-K₁₂₃-G peak. Figure 5 shows the XICs of the two tagged H2B-K₁₂₃ peptides (AVTK₁₂₃SSSTQA) from the wild-type (A) and the Δ Ubp8 yeast strain (B). The H2B peptides are only observed in the +2 charge state. Figure S5 provides MS/MS spectra of both the branched, H2B-K₁₂₃-GG peptide (A) and the derivatized, H2B-K₁₂₃-G peptide (B) observed from glycinylated histone H2B. The *b*- and *y*-ion series in both spectra show the presence of each modified H2B-K₁₂₃ of interest. The H2B-K₁₂₃-GG/(H2B-K₁₂₃-GG+ H2B-K₁₂₃-G) values observed for derivatized histone H2B from wild-type yeast was $2.4\pm0.4\%$ and increased to $17.5\pm1.0\%$ in the Δ Ubp8 strain. This corresponds to a 7.3-fold increase in ubiquitinated histone H2B upon deletion of the DUB responsible for deubiquitinating histone H2B. A densitometric analysis of the α -ubiquitin Western blot (Figure 4) established a 6.2-fold increase of Ub-H2B upon Ubp8 deletion.

Therefore, the glycinylation technique developed here was successfully used to estimate the relative levels of ubiquitinated histone H2B in a wild-type yeast strain versus a DUB knockout yeast strain. The results correlated well with the levels of ubiquitinated histone H2B observed in the Western Blot analysis. For other proteomic applications of the technique, it would be beneficial to synthesize the Gly- and GlyGly-tagged peptide of interest and compare their chemical equivalency with a mass spectrometer to ensure the applicability of the technique to the ubiquitinated site in question.

During the glycinylation reaction, the ubiquitin attached to the isolated histone H2B is also derivatized. Consequently, the same data set can also be interrogated for the K-GG and K-G versions of the lysine-containing ubiquitin peptides to describe the nature of the change in ubiquitination observed; for example, whether it is mono-ubiquitination (lower GG/(GG+G) percent) or poly-ubiquitination (higher GG/(GG+G) percent). The peptides used to search the data will also attest to which ubiquitin linkages are involved with a change in poly-ubiquitination; for example, whether it is the Ub-K₄₈, Ub-K₆₃, or any other ubiquitin peptide.

Figure S6 shows the XICs of the Ub-K₄₈ peptide from the histone H2B samples purified from wild-type (A) and Δ Ubp8 yeast (B). The assignments of these peaks are based on the high mass accuracy in the MS1 scan as well as other data not shown. For example, the retention times of the yeast Ub-K₄₈ peptides match those of the synthetic Ub-K₄₈ peptides. In addition, the sample was also analyzed with a data-dependent method and the MS/MS data confirmed the peptide identities. The signals from the Ub-K₄₈ peptides are weak and the +3 peaks were not of good quality. The low intensity of the peaks resulted in low S/N values and the quantitation measurements have a higher than normal error. Overall, the Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-GG) decreases from an average of $14.6 \pm 5.0\%$ in the wild-type yeast to $1.6 \pm 0.2\%$ in the Δ Ubp8 yeast. The lower Ub-K₄₈-GG/Ub-K₄₈-G value in the DUB knock-out strain is interpreted as higher levels of the derivatized, Ub-K₄₈-G peptide. This shows that the observed increase in ubiquitination of histone H2B is attributable to mono-ubiquitination instead of the formation of poly-ubiquitin chains, which would result in higher levels of the branched, Ub-K₄₈-GG peptide and an increase in the Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-GG) value. This trend was also observed for the Ub-K₆₃ peptide which showed average Ub-K₆₃-GG/(Ub-K₆₃-GG+Ub-K₆₃-GG) of $8.3 \pm 1.6\%$ for the wild-type yeast and $1.6 \pm 0.02\%$ for the Δ Ubp8 yeast (data not shown). A ratio of the change in ubiquitination between the two samples at Ub-K₄₈ (9.1 ± 3.3 fold) and Ub-K₆₃ (5.2 ± 1.0 fold) was calculated to be 1.8 ± 0.7 , showing that a reproducible relative change was observed. The conclusion of an increase in mono-ubiquitination is also consistent with the Western blot analysis (Figure 4) which showed a single shift of approximately 8.5kD instead of a ladder of bands that is indicative of poly-ubiquitination.

Conclusion

The derivatization technique presented here, termed glycinylation, has been developed for the quantitation of ubiquitination. A single glycine tag is used as a structural homologue to the di-glycine tag found at ubiquitination sites after trypsin digestion. The chemical equivalency of the two moieties was compared using the K₄₈-linkage of ubiquitin. The technique was then used to measure the increase in mono-ubiquitinated histone H2B that is observed upon the deletion of the DUB, Ubp8, in yeast. Future work could focus on improving the glycinylation reaction efficiency. For example, denaturing agents could be used to circumvent the problems observed during derivatization due to the tertiary structure of the proteins. A multiple reaction monitoring (MRM) approach could also be developed to improve the accuracy of the quantitation. Furthermore, a stable isotope analog derivative could be created by performing two rounds of synthesis with Boc-¹³C₂, ¹⁵N-G to produce a ¹³C₄, ¹⁵N₂-GG tag.

The potential applications of the glycylation technique are numerous. For example, this method could elucidate the roles of unknown components in the ubiquitin pathway, such as certain E2s, E3s, or DUBs, by quantitating the subsequent change in the levels of an ubiquitinated substrate upon deletion or inhibition of the protein of interest. In addition, this method could be used to identify novel ubiquitination sites by searching mass spectrometric data for peaks with mass shifts of 57 Da. This technique could also be used for the quantitation of ubiquitin-like proteins (UbIs) such as NEDD8 (yeast and human) and ISG15 (human) which also produce a di-glycine tag upon trypsin digestion.¹ However, since the glycylation reaction is dependent on the nature of the protein, the applicability of the method should be proven for each specific application. If the ubiquitination site is known, the two tagged peptides could be synthesized and compared to confirm their chemical equivalency. In addition, a calibration curve for the site of interest could be established to demonstrate linearity and reproducibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. XICs (A) and a mass spectrum (B) from equimolar amounts of the synthetic Ub-K₄₈ peptides, LIFAGK(G)QLEDGR and LIFAGK(GG)QLEDGR

Both peptides have the same retention time and charge states. If the ionization efficiencies of the two peptides were equal, the percent of ubiquitinated protein (Ub-K₄₈-GG/(Ub-K₄₈-GG + Ub-K₄₈-GG), would be 50%; the average calculated Ub-K₄₈-GG/(Ub-K₄₈-GG + Ub-K₄₈-GG) observed is 50.4±1.4%. Therefore, the Gly-tag is an adequate structural analog to the GlyGly-tag for the quantitation of lysine ubiquitination.

Figure 2. A schematic of the glycylation method

Boc-glycine anhydride is used to modify free lysines with a boc-glycine moiety. After deprotection, the boc group is removed and the lysines are labeled with a single glycine tag. The MS signal from the branched peptide of the ubiquitinated form of the protein and the derivatized peptide of the non-ubiquitinated form of the protein are compared to determine the relative amount of ubiquitination.

Figure 3. XICs (A) and a mass spectrum (B) of the two tagged Ub-K₄₈ peptides observed from glycinylated K₄₈-linked di-ubiquitin
The average calculated Ub-K₄₈-GG/(Ub-K₄₈-GG+Ub-K₄₈-GG) observed is 62.0±1.7%.

Figure 4. α -FLAG (A) and α -ubiquitin (B) Western blots of histone H2B isolated from wild-type (WT) yeast (BY4741) and a Δ Ubp8 strain
When the DUB that is responsible for deubiquitinating H2B (Ubp8) is knocked out, the levels of ubiquitinated H2B increase.

Figure 5. XICs of the H2B-K₁₂₃ peptide, AVTK₁₂₃YSSSTQA, from both yeast strains
 The glycylation technique was used to quantitate the extent to which histone H2B is ubiquitinated in a wild-type (WT) yeast strain.(A) compared to a ΔUbp8 strain (B). For the WT strain the observed H2B-K₁₂₃-GG/(H2B-K₁₂₃-GG+H2B-K₁₂₃-G) is $2.4 \pm 0.4\%$ and for the ΔUbp8 strain the observed H2B-K₁₂₃-GG/(H2B-K₁₂₃-GG+H2B-K₁₂₃-G) is $17.5 \pm 1.0\%$. Therefore, a relative 7.3-fold increase in ubiquitinated histone H2B was observed.

Table 1Experimental design for the calibration curve for K₄₈-linked ubiquitin

% of Ubiquitinated Ub-K ₄₈ (Ub-K ₄₈ -GG/(Ub-K ₄₈ -GG+Ub-K ₄₈ -G))	Poly-ubiquitin	Poly-Ub (pmol)	Mono-Ub (pmol)
80%	penta	12	-
75%	tetra	12	-
67%	tri	12	-
50%	di	12	-
33%	di	12	12
25%	di	12	24
20%	di	12	36
17%	di	12	48