Detection of endogenous ubiquitination sites by mass spectrometry has dramatically improved with the commercialization of anti-di-glycine remnant (K-ε-GG) antibodies. Here, we describe a number of improvements to the K-ε-GG enrichment workflow, including optimized antibody and peptide input requirements, antibody cross-linking, and improved off-line fractionation prior to enrichment. This refined and practical workflow enables routine identification and quantification of ~20,000 distinct endogenous ubiquitination sites in a single SILAC experiment using moderate amounts of protein input. Molecular & Cellular Proteomics 12: 10.1074/mcp.O112.027094, 825–831, 2013.

The commercialization of antibodies that recognize lysine residues modified with a di-glycine remnant (K-ε-GG)\(^1\) has significantly transformed the detection of endogenous protein ubiquitination sites by mass spectrometry (1–5). Prior to the development of these highly specific reagents, proteomics experiments were limited to identification of up to only several hundred ubiquitination sites, which severely limited the scope of global ubiquitination studies (6). Recent proteomic studies employing anti-K-ε-GG antibodies have enhanced our understanding of ubiquitin biology through the identification of thousands of ubiquitination sites and the analysis of the change in relative abundance of these sites after chemical or biological perturbation (1–3, 5, 7). Use of stable isotope labeling by amino acids in cell culture (SILAC) for quantification has enabled researchers to better understand the extent of ubiquitin regulation upon proteasome inhibition and precisely identify those protein classes, such as newly synthesized proteins or chromatin-related proteins, that see overt changes in their ubiquitination levels upon drug treatment (2, 3, 5). Emanuel et al. (1) have combined genetic and proteomics assays implementing the anti-K-ε-GG antibody to identify hundreds of known and putative Cullin-RING ligase substrates, which has clearly demonstrated the extensive role of Cullin-RING ligase ubiquitination on cellular protein regulation.

Despite the successes recently achieved with the use of the anti-K-ε-GG antibody, increased sample input (up to ~35 mg) and/or the completion of numerous experimental replicates have been necessary to achieve large numbers of K-ε-GG sites (>5,000) in a single SILAC-based experiment (1–3, 5). For example, it has been recently shown that detection of more than 20,000 unique ubiquitination sites is possible from the analysis of five different murine tissues (8). However, as the authors indicate, only a few thousands sites are detected in any single analysis of an individual tissue sample (8). It is recognized that there is need for further improvements in global ubiquitin technology to increase the depth-of-coverage attainable in quantitative proteomic experiments using moderate amounts of protein input (9). Through systematic study and optimization of key pre-analytical variables in the preparation and use of the anti-K-ε-GG antibody as well as the proteomic workflow, we have now achieved, for the first time, routine quantification of ~20,000 nonredundant K-ε-GG sites in a single SILAC triple encoded experiment starting with 5 mg of protein per SILAC channel. This represents a 10-fold improvement over our previously published method (3).

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**To generate titration curve data, Jurkat E6-1 cells (ATCC) were grown in Roswell Park Memorial Institute (RPMI) 1640 media (Invitrogen) supplemented with 10% dialed fetal bovine serum (Sigma-Aldrich), penicillin, streptomycin, and glutamine (Invitrogen). For titration data where peptide input was held constant and antibody amount was varied, Jurkat cells were treated for 4 h with 2 \(\mu\)g MG-132 (Calbiochem) prior to harvest. For SILAC experiments, Jurkat cells were cultured in RPMI media (custom preparation from Caisson Laboratories, North Logan, UT) deficient in L-arginine and L-lysine and supplemented with 10% dialed fetal bovine serum.
temperature and subsequently carbamidomethylated using 10 mM (DMP) and incubated at room temperature for 30 min while rotating.

Cell Lysis and Trypsin Digestion—SilAC-labeled cell pellets were lysed at 4°C in denaturing conditions. Specifically, the cell lysis buffer contained 8 mM urea, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 μg/ml aprotinin (Sigma-Aldrich), 10 μg/ml leupeptin (Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM PR-619, and 1 mM chloroaceticamide. Following lysis, cells were centrifuged at 20,000 x g for 15 min at 4°C to remove insoluble material. Protein concentrations were estimated using a bicinchoninic acid (BCA) protein assay (Pierce). Each cell lysate was loaded onto conditioned StageTips, washed two times with 100 μl of 100% MeCN, followed by 5 ml of 50% MeCN, 0.1% FA, and eluted with 6 ml of 50% MeCN, 0.1% FA, and finally 20 ml of 0.1% trifluoroacetic acid (TFA). Peptide super-natants were desalted using C18 StageTips (10). Each StageTip column was packed with two C18 Empore™ high performance extraction disks (3 ml) and conditioned with 100 μl of 100% MeCN, 100 μl of 50% MeCN, 0.1% FA, and 2 × 100 μl of 0.1% FA. Peptides were loaded onto conditioned StageTips, washed two times with 100 μl of 0.1% FA, and eluted with 50 μl of 50% MeCN, 0.1% FA. Eluted peptides were dried to completeness.

LC-MS/MS Analysis—For MS analyses, peptides were resuspended in 3% MeCN, 1% FA and analyzed by nanoflow-UPLC-HCD-MS/MS using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled on line to a Proxeon Easy-nLC 1000. Samples were injected onto a microcapillary column (360 μm outer diameter × 75 μm inner diameter) equipped with an integrated electrospray emitter tip (10 mm), packed to 24 cm with ReproSil-Pur C18-AQ 1.9 μm beads (Dr. Maisch GmbH) and heated to 50°C. The HPLC solvent A was 0.1% FA, and the solvent B was 90% MeCN, 0.1% FA. Peptides were eluted into the mass spectrometer at a flow rate of 200 nl/min using a linear gradient of 0.3%B/min, following by a ramp to 60% B (10% B/min). The total run time was 150 min, including sample loading and column conditioning. The Q Exactive was operated in the data-dependent mode acquiring HCD MS/MS scans (R = 17,500) after each MS1 scan (R = 70,000) on the 12 most abundant ions using an MS1 ion target of 3 × 10^4 ions and an MS2 target of 5 × 10^4 ions. The maximum ion time utilized for MS/MS scans was 120 ms; the HCD-normalized collision energy was set to 25; the dynamic exclusion time was set to 20 s, and the peptide match and isotope exclusion functions were enabled.

MS Data Analysis—MS data were analyzed using the MaxQuant (11, 12) software version 1.3.0.5 and searched against the human Uniprot database containing 81,470 entries. A list of 248 common laboratory contaminants provided by MaxQuant was also added to the database. For searching, the enzyme specificity was set to trypsin with the maximum number of missed cleavages set to 2. The precursor mass tolerance was set to 20 ppm for the first search (used for nonlinear mass re-calibration) and was set to 6 ppm for the main search. Oxidized methionines, Gly-Gly addition to lysines, and N-terminal protein acetylation were searched as variable modifications. Carbamidomethylation of cysteines were searched as a fixed modification. The false discovery rate for peptide, protein, and site identification was set to 1%; the minimum peptide length was set to 6, and the filter-labeled amino acid and peptide requantification functions were enabled. K-e-GG site identification and quantification information were obtained from the MaxQuant Gly-Gly sites and evidence tables. For titration curve experiments, identified and nondistinct K-e-GG peptides were grouped based on localization information as described previously (3). For the SILAC-based benchmark experiments, site level information was obtained directly from the MaxQuant Gly-Gly sites table. Sites confidently localized (localization probability of >0.75) to a peptide C-terminal lysine residue were removed from the dataset. Regulated K-e-GG sites were determined using a moderated
T-statistic calculated from the limma package in the R environment as described previously (3, 13). Gene ontology biological process term enrichment was completed using the DAVID bioinformatics resource (14). Supplemental Tables 1 and 2 contain embedded hyperlinks that link to a Spectrum Mill viewer showing an annotated MS/MS spectrum for K-\(\varepsilon\)-GG peptides. We recommend using Internet Explorer on Windows for best results. All raw data are available at ftp://ftp.broadinstitute.org/distribution/proteomics/public_datasets/.

RESULTS

Antibody-derived Contaminants Compromise Detection of Ubiquitinated Peptides—Our previous work with the anti-K-\(\varepsilon\)-GG antibody indicated that contaminant proteins and non-tryptic peptides were frequently present in K-\(\varepsilon\)-GG-enriched samples and were retained on our on-line microcapillary C18 column over multiple runs. These abundant contaminants compromised the detection of lower abundant K-\(\varepsilon\)-GG peptides by electrospray mass spectrometry and often resulted in poor chromatographic peak shapes. After enrichment, K-\(\varepsilon\)-GG-modified peptides are typically eluted from the anti-K-\(\varepsilon\)-GG antibody at low pH using 0.15% TFA (3). Analysis of these eluates indicates that large amounts of light and heavy chain antibody subunits are released from the anti-K-\(\varepsilon\)-GG antibody that is supplied noncovalently bound to protein A beads (Fig. 1a). Furthermore, prior to injection of K-\(\varepsilon\)-GG
peptides from up to 10 mg of starting material and that antibody are also identified after DMP cross-linking (supplemental Fig. 1). Therefore, even after StageTip desalting, abundant non-K-ε-GG species remain in the final samples injected on the mass spectrometer (MS) and hamper the detection of K-ε-GG peptides. In addition, the manufacturer of the anti-K-ε-GG antibody suggests using up to 250 μg of the antibody reagent for each K-ε-GG enrichment experiment. With this level of antibody, the amount of released heavy and light chain fragments surpasses the conventional C18 StageTip capacity by greater than 10x, which likely compromises the binding of K-ε-GG peptides to a C18 StageTip (15).

Cross-linking of Antibody Reduces Interference and Increases Numbers of Detectable K-ε-GG Peptides—To diminish the deleterious effects that antibody-derived contaminants have on the enrichment and detection of K-ε-GG peptides, we evaluated chemical cross-linking of the anti-K-ε-GG antibody to solid support. We find that chemical cross-linking of the antibody to the commercially supplied protein A beads with DMP prevents release of the majority of heavy and light chain fragments upon low pH elution (Fig. 1b) and increases the number of K-ε-GG peptides identified by ~25% (Fig. 1c) (16). This increase is largely independent of the amount of starting anti-K-ε-GG antibody used over a range of 31, 62, 125, or 250 μg when enrichment is completed using 1 mg of protein input (Fig. 1c and supplemental Table 1). We also show that chemical cross-linking of the anti-K-ε-GG antibody does not significantly alter its binding to K-ε-GG peptides because the majority of peptides (62%) identified with noncross-linked antibody are also identified after DMP cross-linking (supplemental Fig. 1).

Ratio of Antibody to Input Lysate has a Large Effect on Enrichment Yield and Specificity—The need to have sufficient antibody to capture all target antigen is generally well understood, but such a study has not been reported for the commercial anti-K-ε-GG antibody. Here, we titrated the amount of anti-K-ε-GG antibody, and in parallel experiments, the amount of input protein derived from cells treated with the proteasome inhibitor MG-132 to study the interplay of these factors on the number of identified K-ε-GG peptides and non-K-ε-GG peptides was identified by LC-MS/MS. Our study revealed that only ~31 μg (1/8 of what is provided for a single assay in the commercial product) of either cross-linked or noncross-linked antibody is needed to achieve the maximum yield of distinct K-ε-GG peptides from 1 mg of digested protein lysate (Fig. 1c and supplemental Table 1). Remarkably, just 31 μg of antibody is sufficient to enrich K-ε-GG peptides from up to 10 mg of starting material and that saturating conditions are achieved only above 10 mg of peptide input (Fig. 1d and supplemental Table 1). As expected from our initial titration experiments (Fig. 1c), the number of identified non-K-ε-GG peptides does not significantly change when antibody input is kept constant and only peptide input is varied (Fig. 1d and supplemental Table 1). Quite unexpectedly, we found that the number of detected K-ε-GG peptides decreased significantly as the input amount of antibody was increased beyond 31 μg. Tracking the number of non-K-ε-GG peptides identified in each experiment revealed that increasing amounts of antibody input steadily increases the number of identified non-K-ε-GG peptides. This indicates a high degree of nonspecific binding of non-K-ε-GG peptides to protein A beads and/or the antibody itself, which impedes detection of K-ε-GG peptides (Fig. 1c). Our results show that it is imperative to determine the optimal antibody and protein input combination to maximize the yield of K-ε-GG peptides in a given experiment. This is likely true for other antibody-based peptide and post-translational modification enrichment protocols.

We further extended our antibody titration study to compare the depth-of-coverage obtainable from 1 mg of protein input derived from cells that had not been treated with MG-132 and therefore had endogenous cellular levels of ubiquitination. Although the number of identified K-ε-GG peptides decreased by ~40% in the absence of proteasome inhibitor, we still successfully identified an average of 5,030 K-ε-GG peptides starting with 1 mg of input and 31 μg of chemically cross-linked antibody (supplemental Fig. 2 and supplemental Table 1). This result indicates that under optimized technical conditions, global ubiquitination assays can be completed in systems that have not undergone prolonged proteasome inhibitor treatment that is important to differentiate ubiquitination sites involved in physiological functions versus those that occur due to stress induced by inhibiting proteasome function (17).

Off-line Basic pH RP Fractionation Increases the Number of Identified K-ε-GG Peptides—Our refined and optimized workflow for deep coverage of K-ε-GG peptides from cellular lysates is shown in Fig. 2. The process is illustrated for a triple-encoded SILAC experiment using 5 mg of input protein per label/condition. In this workflow, SILAC-labeled cells are lysed under denaturing conditions and then combined in equal amounts based on protein content. Following reduction and alkylation, the proteins are digested with trypsin to generate non-K-ε-GG peptides as well as peptides containing the di-glycine remnant. Previously, we used off-line strong cation exchange fractionation to increase the number of K-ε-GG peptides detected by 3–4-fold relative to nonfractionated samples (3). As part of our optimized protocol, we have implemented basic RP chromatography to generate eight total fractions for K-ε-GG enrichment (Fig. 2) (18, 19). We find that basic RP chromatography is superior to our previously published strong cation exchange method because desalting of
peptide fractions is not required prior to K-\(\epsilon\)-GG enrichment as a result of the volatile buffers used, which greatly simplifies sample handling and improves peptide recovery (see under “Experimental Procedures”). Additionally, pooling of nonadjacent basic RP fractions generates highly uniform and nonredundant samples for K-\(\epsilon\)-GG enrichment that enables consistent depth-of-coverage for K-\(\epsilon\)-GG peptides across all eight fractions (Fig. 3b) (18, 19). The eight basic RP fractions are directly enriched for K-\(\epsilon\)-GG peptides using 31 \(\mu\)g of DMP cross-linked anti-K-\(\epsilon\)-GG antibody per fraction (Fig. 2). After enrichment, each fraction was individually analyzed by UPLC-HCD-MS/MS with statistical analysis of the data as described under “Experimental Procedures.”

**Benchmarking of the Optimized Workflow**—We benchmarked the refined and optimized K-\(\epsilon\)-GG workflow using biological samples identical to those described in our previously published work (3). Specifically, SILAC-labeled Jurkat cells were treated for 4 h with either 5 \(\mu\)M of the reversible deubiquitinase inhibitor 2,6-diaminopyridine-3,5-bis(thiocyanate) (PR-619) (20), 5 \(\mu\)M of the proteasome inhibitor MG-132, or were left untreated (Fig. 3a). The new workflow, which employs an optimized amount of chemically cross-linked antibody to enrich K-\(\epsilon\)-GG peptides from eight basic RP fractions, resulted in unparalleled detection of K-\(\epsilon\)-GG sites from a single SILAC triple-encoded sample. We completed three full biological replicates with SILAC label switching, and we found that in a single replicate we quantify an average of 3,143 distinct K-\(\epsilon\)-GG peptides per basic RP fraction and an average of 20,044 distinct K-\(\epsilon\)-GG sites of which 18,133 were confidently localized with a localization probability of \(>0.75\) in total for the eight fractions (Fig. 3, b and c, and supplemental materials).
Table 2). Examples of annotated HCD MS/MS spectra of K-ε-GG-modified peptides are shown in supplemental Fig. 3A, Fig. 3, b and c. This result represents nearly a 10-fold increase in the number of quantified K-ε-GG sites for a single SILAC sample relative to our previously published work where only 2,931 and 1,716 sites were detected and quantified in a single replicate (Fig. 3c) (3). Across three biological replicates, we find that 14,181 K-ε-GG sites are quantified in at least two replicates, and 8,269 sites are quantified in all three replicates (Fig. 3d). Taken together, the marked improvement made in the enrichment and detection of K-ε-GG peptides is achieved through a number of technical enhancements, including the use of optimized antibody input, chemically cross-linked antibody, improved off-line fractionation methods, and advanced MS and LC conditions (supplemental Table 3). Notably, the results reported here far surpass all previously reported yields for quantification and detection of K-ε-GG sites from a single SILAC-labeled sample where starting material is limited to 5 mg per channel (1–3, 5).

Similar to previous findings, we show that proteasome inhibition by MG-132 and deubiquitinase inhibition by PR-619 cause significant changes to the ubiquitin landscape, but MG-132 is more effective at increasing the levels of ubiquitination sites relative to PR-619 (supplemental Figs. 4 and 5) (2, 3). Following cellular treatment with MG-132, ~60% of quantified K-ε-GG sites were reproducibly regulated, whereas ~10% of sites were regulated following PR-619 treatment. With both reagents, we found that significantly up-regulated K-ε-GG sites were enriched on proteins involved in cell cycle progression, whereas down-regulated K-ε-GG sites were enriched on proteins involved in translational regulation. We also noted that our current data corroborate previous findings that proteasome inhibition with MG-132 significantly increases the levels of nearly all polyubiquitin linkage types and is more effective at increasing the levels of polyubiquitin chains than deubiquitinase inhibition by PR-619 (supplemental Fig. 6) (3, 5).

**DISCUSSION**

In summary, we have described a number of critical enhancements to the K-ε-GG analysis pipeline that, taken together, have resulted in the most robust, sensitive, and practical method described to date for the analysis of endogenous ubiquitination sites from cellular lysates. The specific improvements we have introduced, including cross-linking of the antibody, optimization of the amount of antibody needed

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**Workflow for Quantification of 10,000s of K-ε-GG Sites**

**Fig. 3. a,** description of SILAC-labeling states and corresponding drug treatment conditions for biological replicate 1 of the SILAC benchmarking study. **b,** number of distinct K-ε-GG and non-K-ε-GG peptides identified per basic RP fraction across three biological replicates. Approximately 90% of K-ε-GG peptides are unique to a single basic RP fraction. **c,** number of distinct K-ε-GG sites quantified in our benchmarking study relative to the number of sites quantified in two biological replicates from our previous work (3). **d,** Venn diagram showing the overlap of quantified K-ε-GG sites across three biological replicates.
for efficient enrichment of K-ε-GG-peptides, as well as use of peptide separation methods that do not require desalting of sample prior to analysis by LC-MS/MS, all lend themselves to future automation of the technology for parallel processing of many samples simultaneously. We have shown, for the first time, that the depth-of-coverage obtainable from moderate amounts of starting material is on par with the degree of coverage obtained using highly optimized methods to detect protein phosphorylation sites (21, 22), moving the field closer to the goal of detecting and quantifying even low level ubiquitin sites from limiting amounts of protein input using the anti-K-ε-GG antibody (9). We note that further increases in the number of ubiquitination sites detected and quantified in single experiments are possible, but they will likely require the use of more than 5 mg of input protein per label state combined with further decreases in sample complexity, for example by use of two-state versus three-state SILAC, or by peptide level fractionation into a larger number of fractions prior to enrichment. We anticipate that the techniques and refinements described in this work will not be limited in usefulness to only experiments using the anti-K-ε-GG antibody but will also be highly valuable in the future for optimization of other post-translational modification-specific antibodies.

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Competing Financial Interests

The authors declare no competing financial interests.

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