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Analysis of the biochemical role of Lys-11 in polyubiquitin chain formation using quantitative mass spectrometry

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RATIONALE: Protein ubiquitination plays a critical role in regulating many cellular events, such as protein localization and stability, cellular signal transduction and DNA repair. Recent studies have shown that polyubiquitin (polyUb) chains elongate through heterogeneous isopeptide linkages to K11, K29, K48 and K63. In this study we have investigated the usage of isopeptide linkages of polyUb chains in different molecular weight regions by using quantitative mass spectrometry.
METHODS: Recombinant Chfr protein was autoubiquitinated by E1 enzyme, E2 enzyme UbcH5 and ubiquitin (WT Ub, K11R Ub, K48R Ub and K63R Ub) *in vitro*, and different molecular weight regions of ubiquitinated Chfr were then subjected to liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) following sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digestion.
RESULTS: Absolute QUantitative Analysis (AQUA) of polyUb chain formation with wild-type (WT) and point mutants of ubiquitin was performed, and the results suggested that the K11 polyUb chain was most frequently used in the high ubiquitin conjugates of WT Ub. Furthermore, the extent of polyUb chain formation with K11R Ub was decreased about 10-fold compared to polyUb chain formation with WT Ub through the entire molecular weight region. The present study suggests that the linkage through K11 plays crucial roles in polyUb chain formation.
CONCLUSIONS: Topologies of polyUb chains in the low and high Ub conjugates were studied using mass spectrometry. K48 and K63 were the primary ubiquitination sites of the low molecular weight Ub conjugates, whereas K11 was the critical site of polyUb chain formation in high molecular weight Ub conjugates. Copyright © 2012 John Wiley & Sons, Ltd.

Ubiquitin (Ub) is a small regulatory protein that is conjugated to target proteins for proteasomal degradation,[1–3] regulation of endocytosis[4] and protein localization,[5] and initiating cell signaling, DNA repair and gene transcription.[6–11]

Ubiquitination is a protein post-translational modification, which is the covalent attachment of one or more Ub monomers to the substrate proteins by formation of an isopeptide bond between the ε-amino group of substrate lysines or a lysine residue of Ub itself and C-terminal carboxyl group of a Ub. Ubiquitination occurs through a series of coordinated actions of Ub-activating enzyme (E1), Ub-carrier proteins (E2) and a Ub-ligase (E3).[2,12–14]

The regulation of a variety of functions of Ub is dependent on the extent of ubiquitination and the topology of the polyUb chains.[5] So far, K48-linked polyUb chains have been experimentally proven to target substrates for proteasomal degradation, while K63-linked polyUb chains have been shown and cataloged to regulate DNA repair,[6,15] signal transduction and protein trafficking. A recent report has suggested that polyUb chains linked mainly through K63 on EGFR target the substrate protein to the lysosome.[16]

Recent studies using tandem mass spectrometric (MS/MS) methods have shown that polyUb chains are also linked through K6, K11, K27, K29 and K33 as well as K48 and K63, although the function of these polyUb linkages in cellular events has not clearly been shown.[17–20] Hence, deciphering molecular mechanisms of polyUb chain formation is of a great importance for the detailed understanding of sequential conjugation of Ub during polyUb chain formation.

Recently, biochemical and biological functions of particular polyUb chain linkages were investigated. It was shown that the formation of particular polyUb chains was closely associated with specific E2s and the E2-E3 combinations. Several groups have shown that the specific E2-E3 combination of APC E3 and UbcH10 E2 facilitates the formation of the K11-linked polyUb chain.[21,22] Furthermore, Peng *et al*. have demonstrated that Ubc6 primarily synthesized K11-linked

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polyUb chains and K11 linkages had a critical role in the endoplasmic reticulum-associated degradation (ERAD) pathway in yeast.[23] In addition, both K11- and K48-linked polyUb chains served as targeting signals for the protreasome.

In this study, we investigated the molecular events of *in vitro* polyUb chain formation with Chfr (checkpoint protein with FHA and RING domains) in the presence of E1, E2 and Ub. Chfr is an E3 Ub ligase that targets Polo-like kinase 1 (Plk1) for degradation *in vivo* and catalyzes its own ubiquitination *in vitro*. The ring finger domain in Chfr is required for the ligase activity. Ubiquitination of Plk1 by Chfr delays activation of the Cdc25C phosphatases and inhibits Cdc2 at the G2 to M transition.

Our study showed that the polyUb chains of autoubiquitinated Chfr were linked mostly through K11, K48 and K63 with different degrees of usage, depending on the extent of the polyUb chain, and the biochemical roles of the lysine residues in polyUb chain formation were confirmed by Western blot analysis and mass spectrometry (MS) techniques.

Although the physiological significance of polyUb chain formation through K11 still remains an open question, it is quite possible that polyUb chain formation through K11 is required for propagation of polyUb chain formation which is initiated through K48 or K63. Further studies are needed to investigate how polyUb chain formation through K11 regulates polyubiquitination.

EXPERIMENTAL

Materials

All chemicals were obtained from Sigma (St. Louis, MO, USA), unless specifically indicated otherwise.

In vitro ubiquitination

For *in vitro* ubiquitination assays, His-Chfr (3 μg) purified from insect cells was incubated at 37°C for 30 min with E1 (2 μg) (BostonBiochem, MA, USA), UbcH5 (2 μg), Ub (WTUb, K11RUb, K48RUb and K63RUb; 5 μg; BostonBiochem, MA, USA) and an ATP-regenerating system (50 mM Tris–HCl at pH 7.5, 5 mM $MgCl_2$, 10 mM creatine phosphate, 5 U/mL phospho-creatine kinase, 5 mM ATP and 1X protease inhibitor cocktail). After incubation, ubiquitinated Chfr was affinity purified by Ni column chromatography followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) separation of the Chfr according to their degree of ubiquitination.

SDS-PAGE and Western blotting

One-dimensional SDS-PAGE was applied. Ubiquitinated Chfr samples were loaded in a single lane on a 1.5 mm thick 4–12% Tris-glycine gel (Invitrogen, CA, USA) and 1.5 mm thick 3–8% Tris-acetate gel (Invitrogen, CA, USA). After separation, the gel was stained with GelCode Blue Stain Reagent (Pierce, IL, USA). Separated proteins on SDS-PAGE gel were transferred onto nitrocellulose membranes (Schleicher & Schuell BioScience, Dassel, Germany). These membranes were blocked with 3% non-fat dry milk (BD, MD, USA) and 0.2% Triton X-100 in Tris-buffered saline (TBS), and subsequently probed with primary antibody in TBS which contained 3% non-fat dry milk and 0.2% Triton X-100. The antibody-antigen complexes were then detected using mouse anti-mouse IgG peroxidase conjugates, followed by the use of a home-made enhanced chemiluminescence (ECL) solution.

Enzymatic in-gel digestion

Coomassie blue stained gel containing the band of Chfr and the upper ubiquitnated bands were sliced into sizes of ~1 mm^3 and transferred to a clean microcentrifuge tube. Gel pieces were destained with 50% acetonitrile (ACN) containing 50 mM NH_4HCO_3, dehydrated with ACN and dried by SpeedVac. Sequential in-gel digestion was carried out overnight with 12.5 ng/mL sequencing-grade modified trypsin (Promega, WI, USA) in 50 mM NH_4HCO_3 buffer (pH 7.8) at 37°C followed by digestion with Glu-C (Roche, Basel, Switzerland) in 25 mM NH_4HCO_3 buffer (pH 7.8) at 25°C. The digested peptides were then extracted with 5% formic acid in 50% ACN solution at room temperature for 20 min. The supernatants were collected after centrifugation and dried by SpeedVac. The samples were purified using C18 ZipTips (Millipore, MA, USA) before MS analysis.

Nano-LC/ESI-MS/MS analysis

The digested peptides were loaded onto a trapping column. After washing with buffer A, the peptides ware separated by high-performance liquid chromatography (HPLC) using an Ultimate 300 system (Dionex LC Packings, Idstein, Germany) with a linear gradient of 3–55% buffer B in 75 min followed by 55–90% buffer B in 15 min (buffer A: 0.1% formic acid in H_2O, buffer B: 0.1% formic acid in ACN) at a flow rate of 250 nL/min. The same LC conditions were used for both data-dependent analysis and AQUA experiments. The fused silica microcapillary column (12 cm × 75 μm) which was packed with C18 resin (5 μm, 300 Å) was connected directly to the LTQ ion-trap mass spectrometer (ThermoFinnigan, CA, USA). The electrospray voltage was set at 2.0 kV, and the threshold for switching from MS to MS/MS was 250. The normalized collision energy for MS/MS was 35% of main radiofrequency (RF) amplitude, and the duration of activation was 30 ms. All spectra were acquired in data-dependent mode. Each full MS scan was followed by nine MS/MS scans, corresponding to the most intense peak to the ninth most intense peak of the full MS scan. Repeat count of peak for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was set at 180 s, and exclusion mass width was ±1.5 *m/z* units. The list size of dynamic exclusion was 50.

Data analysis

Data were processed and database searched using the SEQUEST algorithm (ThermoFinnigan, CA, USA). Data were processed using TurboSEQUEST (Thermo Electron) which was used with precursor and product ion mass tolerances of 1.5 and 1 *m/z* units, respectively, and all the processed MS/MS spectra were searched against the small database, including Chfr and Ub proteins. Two missed cleavages allowed for trypsin and Glu-C, a fixed carbamidomethyl modification for Cys (+57 *m/z* units), variable oxidation modification for Met (+16 *m/z* units), and ubiquitination modification for Lys

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(+114.1 *m/z* units) were specified. After searching with the combined peptide sequences, Xcorr must be more than 1.8, 2.2 and 3.3 for the + 1, + 2 and + 3 charge states, respectively, with deltaC$_n$ greater than 0.1.

Quantification of ubiquitin chains on ubiquitin-Chfr conjugates

In order to quantify the polyUb chain, isotope dilution analysis was performed for three ubiquitinated peptides of Ub (Table 1). Isotopically labeled ubiquitinated peptides modified at K11, K48 and K63 were synthesized as internal standards (Anygen, Gwangju, Korea). Quantification of the amount of each internal standard peptide was performed by amino acid analysis (KBSI, Daejon, Korea). SDS-PAGE gel regions corresponding to modified Chfr were digested sequentially with trypsin and Glu-C in the presence of internal standard peptides. LC-selected reaction monitoring (SRM) data were acquired with product ion mass tolerance of ±1.5 *m/z* units and with determined SRM transitions and respective retention times for both light and heavy peptides[24,25] as summarized in Table 1. Data were processed by integrating the appropriate peaks for the native and internal standard peptides, followed by calculation of the ratio of peak areas to estimate the abundance of native peptides.

RESULTS AND DISCUSSION

In vitro ubiquitination of Chfr

To characterize the topology of polyUb chain linkages generated by Chfr, *in vitro* ubiquitination of Chfr was performed in the presence of E1 and E2 UbcH5 that are partners of Chfr and wild-type (WT) or mutant Ubs as indicated. After *in vitro* ubiquitination, the reaction mixtures were fractionated by SDS-PAGE, visualized by Coomassie blue staining and Western analysis. As shown in Figs. 1(a) and 1(b), smearing band patterns in the high molecular weight region were noted in the lanes of WTUb, K48Rub and K63Rub, whereas the control Chfr protein migrated at the expected molecular weight of approximately 73 kDa. The smearing bands in a Coomassie blue stained gel run (Fig. 1(a)) and immunoblotting with Chfr Ab (Fig. 1(b)) clearly indicated that Chfr catalyzes self-ligation of Ub molecules.

The Ring domain E3 Ub ligases with E2 UbcH5 have been reported to generate polyUb chains linked with various lysines, mostly K11, K48 and K63 in Ub.[18,19] Therefore, Chfr which belongs to the Ring domain E3 Ub ligase was also expected to form polyUb chains with isopeptide linkages.

To characterize the forms of Ub chains conjugated to Chfr according to the extent of polyubiquitination, high molecular weight gel regions based on the molecular weight of Ub conjugates were subjected to in-gel digestion with trypsin and Glu-C and microcapillary LC/MS/MS. Database searching of the acquired MS/MS spectra identified various ubiquitinated signature peptides from Chfr and Ub; trypsin digestion of Ub-conjugated protein produced the signature peptide with a Gly-Gly tag covalently attached to ubiquitinated lysines on the substrate protein, leading to a characteristic ubiquitination mass shift (+114.1 *m/z* units) on the lysine residue). With the signature mass, LC/MS/MS analyses of samples from in-gel digestion clearly indicated that the polyUb chains conjugated to the Chfr protein were composed of heterogeneous branches linked mostly through K48, K63 and K11 with minor species of polyUb chain formation via K6, K6/11 and K33. The heterogeneous polyUb chain formations were estimated semi-quantitatively. Although the mass spectrometric signal for any given peptide in an LC/MS/MS experiment is not directly correlated with the amount of peptide present in the sample, the number of spectra sequenced to the corresponding peptide is indicative of the relative abundance of the peptide in a limited dynamic range.[18,26] As shown in Table 2, we used a peptide spectral counting method (peptide detection frequency) for the estimation from the LC/MS/MS database search results. The extent of polyUb chain formation with K11RUb was reduced significantly but not with K48R and K63R Ubs.

As the Chfr protein catalyzes its own ubiquitination as well as ubiquitination of Plk1 leading to its degradation,[27,28] the polyUb chains synthesized by the Chfr protein were expected to be linked mainly through K48. In the case of using WT Ub, however, data analysis of the low molecular weight region (L) indicated that less than seven Ub molecules were conjugated to a Chfr and the majority of Ub chains were linked through K48 and K63 with similar frequencies. K6- and K11-linked chains were also detected, but with very low frequencies. Interestingly, in the middle molecular weight region (M) where the number of Ub molecules conjugated to a Chfr was between 8 and 20, chains linked through K63 became the most frequently used, while K6- and K11-linked polyUb

Table 1. Detailed information of ubiquitinated peptides for quantification by AQUA

Ubiqitinated peptide	Type	Sequence	Precursor ion (*m/z*)	Product ions (*m/z*) First	Product ions (*m/z*) Second
K11	Isotope	TLTGK@TI*TLE	599.4.	983.4	882.3
	Wild-type	TLTGK@TITLE	595.9	976.4	875.3
K48	Isotope	LIFAGK@QL*E	570.4	913.3	695.3
	Wild-type	LIFAGK@QLE	566.9	906.3	688.3
K63	Isotope	TLSDYNI*QK@E	666.5	1117.3	942.3
	Wild-type	TLSDYNIQK@E	663.0	1110.3	935.3

K@: Ubiquitinated site K; X*: Isotope labeled X.

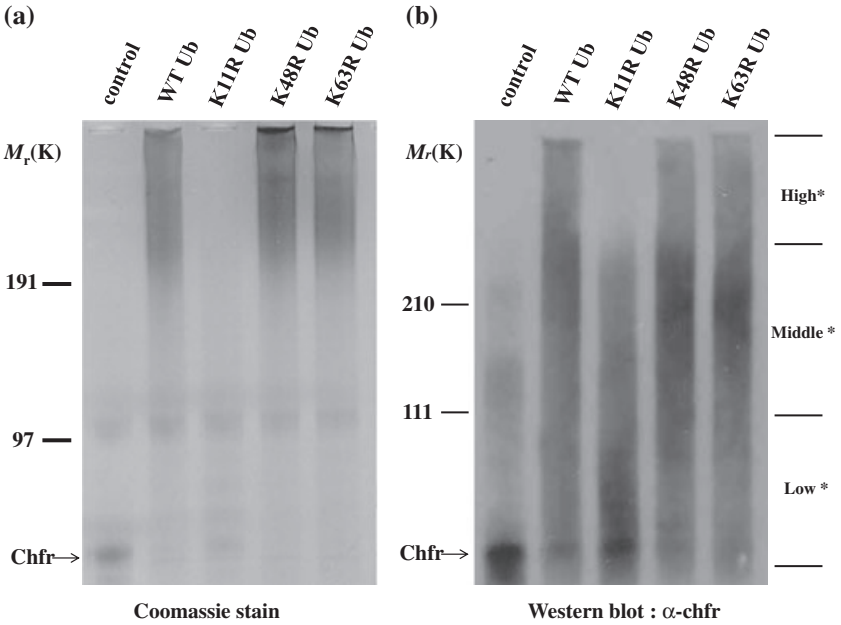

Figure 1. *In vitro* autoubiquitination was performed in the presence of E1, UbcH5 as the E2, ATP and wild-type or mutant Ub as indicated. (a) Ubiquitinated Chfr was separated by SDS-PAGE and stained by Coomassie blue. The extent of ubiquitination in the presence of K11R Ub was dramatically reduced; especially, polyubiquitination with high molecular weight was mostly affected. (b) Western blotting using anti-Ub antibody showed the same result as that of K11R Ub group with low density. Each of these gels represents a typical result obtained by three independent experiments.*Molecular weight region of the gel.

Table 2. Frequencies of detected ubiquitinated peptides. Ubiquitination of Chfr was performed in the presence of wild-type (WT) or mutant Ub as indicated. The extent of polyUb chain formation with K11RUb was reduced significantly but not with K48R and K63R Ubs

Molecular weight region of Gel	PolyUb Site	Number of Ub linkages WT	K11R	K48R	K63R
Low	K11	+	ND	+	+
	K48	+	+	ND	+
	K63	+	+	+	ND
Middle	K11	+	ND	+	+
	K48	+	+	ND	+
	K63	++	+	++	ND
High	K11	+++	ND	+++	+++
	K48	+++	+	ND	+++
	K63	+++	+	+++	ND

ND: not detected.
Filtering: DeltaCn >0.1; Xcorr = +1 > 1.8, +2 > 2.2, +3 > 3.3, number of top match = 1
Modifications: M = 16, C = 57, K = 114.1
[+]indicates low frequencies, i.e. <50; ++ indicates moderate frequencies, i.e. 50 < frequencies < 150; +++ indicates high frequencies, i.e. >150

chains were still minor species. In the high molecular weight region (H), in which more than 21 Ub molecules were conjugated to a Chfr, K11 linkages became one of the major species, whose frequency was almost equal to those of K48 and K63, while K6/11- and K33-linked chains were also detected with low frequencies (data not shown). The relative order of frequencies for lysine usage was K63 ≈ K48 ≈ K 11 > > K6 ≈ K6/11 ≈ K33 (Table 2). These results suggest that the topology of the chains varies depending on the extent of polyubiquitination. As summarized in Table 2, the polyUb chains both in the lower and the middle molecular weight gel regions were linked mostly through K48 and K63, whereas the high molecular weight regions of polyUb chains were composed of K11, K48 and K63 as major species while K6, K6/11 and

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K33 were minor species. Taken together, these findings indicate that Chfr synthesizes heterogeneous polyUb chains with UbcH5, which are conjugated mainly with K11, K48 and K63.

To investigate the functional roles of the isopeptide linkages in polyUb chains generated by the combination of UbcH5 and Chfr, we used mutant Ubs in which the lysine residues had been mutated to arginines: K11R, K48R and K63R. Interestingly, polyUb chain formation was significantly reduced throughout the entire gel regions (H, M and L) (shown in Fig. 1) only when K11R Ub was used instead of WT Ub (Fig. 1). The polyUb conjugates formed with K11R Ub substantially reduced both molecular weight and efficiency of ployUb chain formation. In contrast, immunoblotting analysis revealed that the Chfr could assemble polyUb conjugates even without K48 or K63 in Ub, shown by the fact that the polyUb chain formation with K48R and K63R Ubs resulted in no significant decrease of the chain formation (Fig. 1). As shown in Table 2, quantitative analysis of polyUb chain formation suggested that the mutation of K48 or K63 of Ub was reversed by the use of other available lysine residues, such as K11 and K63 or K48. Thus, these results suggest that Chfr/UbcH5-mediated polyUb chain formation absolutely requires K11, but not K6 or K33.

These results corroborate previous findings showing a preference for K11 linkages in polyUb chains generated by APC with E2 proteins, UbcH10 or UbcH5a.[22] It has been

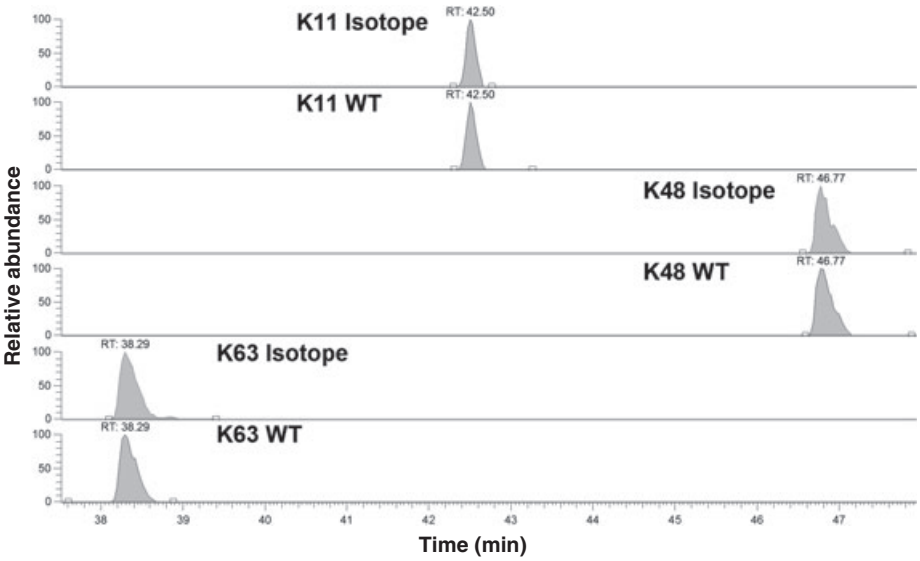

Figure 2. Extracted ion chromatogram of MRM transitions of isotope and WT ubiquitinated peptides. Data were processed by integrating the appropriate peaks for the isotope and WT peptides, followed by calculation of ratio of peak areas to estimate the abundance of the peptides.

Table 3. Amount of detected ubiquitinated peptides. Ubiquitination of Chfr was performed in the presence of wild-type (WT) or mutant Ub as indicated. The amounts of ubiquitinated peptides were determined by AQUA. The extent of polyUb chain formation with K11RUb was reduced significantly

Molecular weight region of gel	PolyUb Site	Amount of Ub linkage (pmol) WT	K11R	K48R	K63R
Low	K11	0.09	NQ	0.51	0.32
	K48	0.35	0.15	NQ	0.76
	K63	0.43	0.27	0.94	NQ
	SUM	0.87	0.42	1. 5	1.1
Middle	K11	0.64	NQ	1.2	0.74
	K48	0.72	0.41	NQ	1.4
	K63	0.76	0.38	1.5	NQ
	SUM	2.1	0.79	2.7	2.1
High	K11	10.8	NQ	11.8	5.2
	K48	8.2	0.65	NQ	7.1
	K63	6.6	0.54	6.7	NQ
	SUM	25.5	1.2	18.5	12.2

NQ: not quantifiable

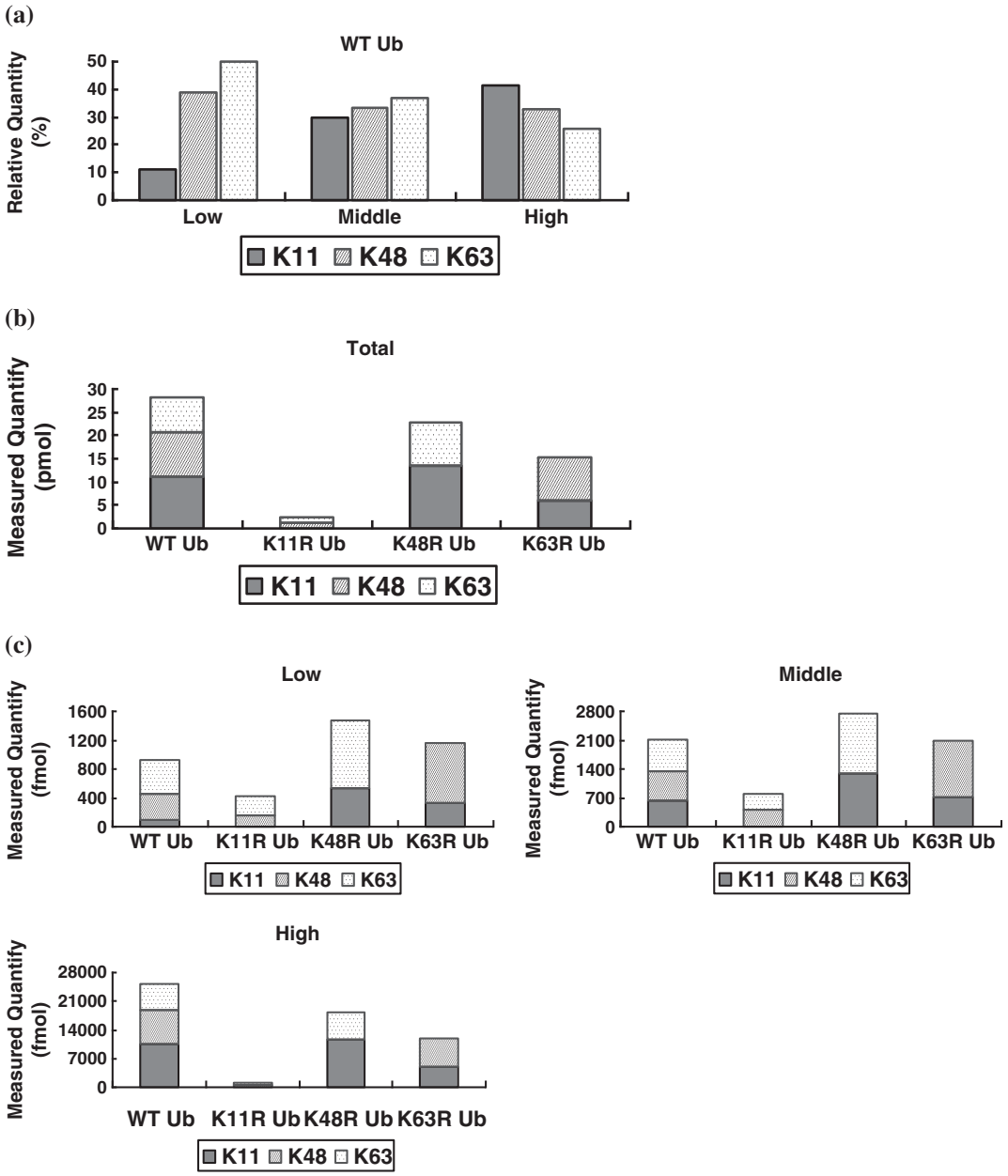

Figure 3. Results of AQUA. The quantitative analysis of the usages of lysine residues in polyUb chain formation. (a) In the case of WT Ub, ratios (K11:K48:K63) of the relative usages of lysine residues in polyUb chain formation changed as follows; 1.0:4.0:4.9 (L) → 1.0:1.1:1.2 (M) → 1.0:0.7:0.6 (H). (b) Abundance of polyUb chain using K11R Ub was very low compared to other Ubs. (c) Polyubiquitination inhibitions were consistently observed in the low, middle and high molecular weight regions, when incubated with K11R Ub. These results were averaged by duplicated experiments.

well documented that Ub linkages through K48 and K63 are major species during polyUb chain formation; therefore, ubiquitinations with K48R or K63R Ubs are expected to reduce polyUb chain formation. However, the dramatic decrease in polyUb chain formation with K11R suggested the critical role that K11 plays in high molecular weight polyUb chain formation mediated by UbcH5 and Chfr, which was further confirmed by MS analysis, as shown in Table 2. It also suggested that K48R and K63R Ubs facilitated polyUb chain formation mediated by UbcH5 and Chfr. Taken together, these results suggest that K48 and K63 are not absolutely required for the polyUb chain formation mediated by Chfr: with K48R and K63R Ubs, Chfr generated polyUb chains with length and topology similar to those generated with WT Ub.

Quantification of ubiquitin linkages

According to the peptide detection frequency based on spectral counts, the usage of K11 in polyUb chain formation has been shown to be as frequent as those of K48 and K63 linkages. However, detection by frequency is not fully quantitative because the ionization efficiency of peptides with

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different amino acid compositions and sequences can vary over several orders of MS signal intensities. Therefore, the quantification of Ub linkages was further validated by an isotope dilution mass spectrometric method, AQUA.[19,25] Table 1 shows a list of heavy labeled peptides that were synthesized based on the identified peptides corresponding to the Ub linkages. These synthetic peptides were used to detect and quantify the target peptides. To determine the exact amount of ubiquitin branches formed by *in vitro* autoubiquitination of Chfr, the reaction mixture of ubiquitination (20 μg) was resolved by a SDS gel, and the gel was excised according to molecular weights of Ub conjugates of Chfr and digested with trypsin and Glu-C spiked with the synthetic peptides(0.5 pmol each). During the quantitative analysis based on the AQUA method,[25] the targeted peptide pair was isolated and subjected to fragmentation by collision-induced dissociation, as shown in Table 1. Each pair of product ions was detected and quantified. As shown in Fig. 2, those ubiquitinated peptides were quantified by isotope dilution analysis, and the amounts of ubiquitinated peptides were determined against internal standard peptides added to the enzymatic digest. Table 3 shows that the results of quantitative analysis appeared to be consistent with previous experiments quantified by the frequencies of detected peptides. Interestingly, K11 was most abundant in the high molecular weight region (40% of the total ubiquitin branches) among the three ubiquitin branches quantified in polyUb conjugates which were formed with WT Ub. Consistent with the results shown by the frequencies of ubiquitinated peptides detected, the usages of K11 progressively increased as the molecular weights of Ub conjugates increased.

As shown in Fig. 3(a), analysis of the low molecular weight region showed that Ub chains were linked mainly through K48 and K63: K11 (11%), K48 (39%) and K63 (50%). In the middle molecular weight region, a significant increase in the linkages through K11 was observed, reaching to 30% of the total Ub linkages, whereas the linkages through K48 and K63 accounted for 33% and 37% of the total Ub linkages, respectively (Fig. 3(a)). In contrast, K11 polyUb chain formation was used most frequently in the high molecular weight region (Fig. 3(a)).

Taken together, in *in vitro* ubiquitination with E1, E2 UbcH5, Chfr and WT Ub, the relative order of lysine usages in polyUb conjugates changed as follows: $K63 > K48 >> K11$ (low molecular weight) $\rightarrow K63 \approx K48 > K11$ (middle molecular weight) $\rightarrow K11 > K48 > K63$ (high molecular weight) (Fig. 3(a)). Furthermore, the abundance of polyUb chains using K11R Ub was very low compared to those formed using other Ubs (Fig. 3(b)): Ubiquitination of K11R Ub was reduced by ~90% as compared to WT Ub, whereas ubiquitinations of K48R or K63R Ubs displayed stronger ubiquitination (about 80% or 40% compared to WT Ub) (Fig. 3 and Tables 2 and 3).

These results suggest that K11 may play a critical role in the high molecular weight polyUb chain formation, since a dramatic decrease in polyUb chain formation in the medium and high molecular weight regions was observed only with K11R Ub: more than 53%, 63% and 95% inhibitions were consistently observed in the low, middle and high molecular weight regions, respectively, when incubated with K11R Ub (Fig. 3(c)).

LC/MS/MS analysis revealed the heterogeneous nature of polyUb chain linkages on Chfr, consistent with recent studies that polyUb chain linkages are composed of several different Ub linkages, such as K11 and K63 as well as K48.[17–21] Especially, the degradation signal on cyclin B1 which is mediated through APC^{cdc20} consists primarily of K11, K48 and K63 Ub linkages with very few of K29 and K33. Furthermore, the topology and relative abundance of Ub linkages vary according to E2 and the extent of polyubiquitination during *in vitro* ubiquitination.[19] They reported that APC-UbcH10 resulted in 1.0:0.2:0.4 (K11:K48:K63) Ub linkages, respectively, whereas APC-Ubc4 produced 1.0:0.8:0.7 (K11:K48:K63) Ub linkages in the high molecular weight region of the resulting polyUb conjugate. In addition, through APC-UbcH10 which assembles the heterogeneous polyUb chain involved in a large majority of K11 Ub chain linkages, they trigger degradation of their substrates.[21]

In contrast, however, Chfr-UbcH5 results in 1.0:0.8:0.7 (K11:K48:K63) linkages and very few of K6 and K6/11 in the corresponding high molecular weight region during *in vitro* ubiquitination (Fig. 3(a)). Together with APC-UbcH10 and APC-Ubc4 systems,[19] this result suggests that E3 in combination with E2 regulates the topology and the frequency of Ub-Ub linkages. Bish *et al*. reported that Werner helicase interacting protein 1 (WRNIP1) was heterogeneously polyubiquitinated *in vivo* through K11, K48 and K63, demonstrated by using a tandem affinity purification technique coupled with mass spectrometry.[20]

CONCLUSIONS

The topologies of polyUb chains in the low and high Ub conjugates were studied using mass spectrometry. In order to analyze the topology of polyUb chains conjugated to the Chfr protein, SDS-PAGE gel regions were excised according to the extent of ubiquitination and subjected to in-gel enzymatic digestion. The resulting enzymatic peptides were analyzed by LC/MS/MS. For more accurate quantitative analysis of ubiquitinated peptides, isotope dilution analysis was applied. The results showed that K48 and K63 were the primary ubiquitination sites of the low molecular weight Ub conjugates, whereas K11 was the critical site of polyUb chain formation in high molecular weight Ub conjugates from *in vitro* ubiquitination mediated by Chfr-UbcH5.

Acknowledgements

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REFERENCES

[1] A. L. Goldberg. Protein degradation and protection against misfolded or damaged proteins. *Nature* **2003**, *426*, 895.
[2] A. Hershko, A. Ciechanover. The ubiquitin system. *Annu. Rev. Biochem.* **1998**, *67*, 425.
[3] C. Picart, C. Gergely, Y. Arntz, J. C. Voegel, *et al*. Measurement of film thickness up to several hundreds of nanometers using optical waveguide lightmode spectroscopy. *Biosensors & Bioelectronics* **2004**, *20*, 553.
[4] L. Hicke, H. Riezman. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. *Cell* **1996**, *84*, 277.

[5] M. Li, C. L. Brooks, F. Wu-Baer, D. Chen, *et al*. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science (New York, N.Y.)* **2003**, *302*, 1972.
[6] J. Spence, S. Sadis, A. L. Haas, D. Finley. A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol. Cell. Biol.* **1995**, *15*, 1265.
[7] E. S. Johnson, P. C. Ma, I. M. Ota, A. Varshavsky. A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J. Biol. Chem.* **1995**, *270*, 17442.
[8] C. Hoege, B. Pfander, G. L. Moldovan, G. Pyrowolakis, S. Jentsch. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **2002**, *419*, 135.
[9] L. Gregori, M. S. Poosch, G. Cousins, V. Chau. A uniform isopeptide-linked multiubiquitin chain is sufficient to target substrate for degradation in ubiquitin-mediated proteolysis. *J. Biol. Chem.* **1990**, *265*, 8354.
[10] L. Deng, C. Wang, E. Spencer, L. Yang, *et al*. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* **2000**, *103*, 351.
[11] V. Chau, J. W. Tobias, A. Bachmair, D. Marriott, *et al*. A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science (New York, N.Y.)* **1989**, *243*, 1576.
[12] C. M. Pickart. Back to the future with ubiquitin. *Cell* **2004**, *116*, 181.
[13] A. Hershko, H. Heller. Occurrence of a polyubiquitin structure in ubiquitin-protein conjugates. *Biochem. Biophys. Res. Commun.* **1985**, *128*, 1079.
[14] M. Hochstrasser. Ubiquitin-dependent protein degradation. *Annu. Rev. Genetics* **1996**, *30*, 405.
[15] R. M. Hofmann, C. M. Pickart. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **1999**, *96*, 645.
[16] F. Huang, D. Kirkpatrick, X. Jiang, S. Gygi, A. Sorkin. Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol. Cell* **2006**, *21*, 737.
[17] J. Peng, D. Schwartz, J. E. Elias, C. C. Thoreen, *et al*. A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* **2003**, *21*, 921.
[18] H. T. Kim, K. P. Kim, F. Lledias, A. F. Kisselev, *et al*. Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. *J. Biol. Chem.* **2007**, *282*, 17375.
[19] D. S. Kirkpatrick, N. A. Hathaway, J. Hanna, S. Elsasser, *et al*. Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. *Nat. Cell Biol.* **2006**, *8*, 700.
[20] R. A. Bish, O. I. Fregoso, A. Piccini, M. P. Myers. Conjugation of complex polyubiquitin chains to WRNIP1. *J. Proteome Res.* **2008**, *7*, 3481.
[21] L. Jin, A. Williamson, S. Banerjee, I. Philipp, M. Rape. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell* **2008**, *133*, 653.
[22] T. Wu, Y. Merbl, Y. Huo, J. L. Gallop, *et al*. UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 1355.
[23] P. Xu, D. M. Duong, N. T. Seyfried, D. Cheng, *et al*. Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* **2009**, *137*, 133.
[24] L. Anderson, C. L. Hunter. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Mol. Cell. Proteomics* **2006**, *5*, 573.
[25] S. A. Gerber, J. Rush, O. Stemman, M. W. Kirschner, S. P. Gygi. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6940.
[26] H. Liu, R. G. Sadygov, J. R. Yates 3rd. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76*, 4193.
[27] E. Shtivelman, Promotion of mitosis by activated protein kinase B after DNA damage involves polo-like kinase 1 and checkpoint protein CHFR. *Mol. Cancer Res.* **2003**, *1*, 959.
[28] D. Kang, J. Chen, J. Wong, G. Fang. The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. *J. Cell Biol.* **2002**, *156*, 249.

