Polyubiquitin Linkage Profiles in Three Models of Proteolytic Stress Suggest the Etiology of Alzheimer Disease*

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Polyubiquitin chains on substrates are assembled through any of seven lysine residues or the N terminus of ubiquitin (Ub), generating diverse linkages in the chain structure. PolyUb linkages regulate the fate of modified substrates, but their abundance and function in mammalian cells are not well studied. We present a mass spectrometry-based method to measure polyUb linkages directly from total lysate of mammalian cells. In HEK293 cells, the level of polyUb linkages was found to be 52% (Lys48), 38% (Lys63), 8% (Lys29), 2% (Lys11), and 0.5% or less for linear, Lys6, Lys27, and Lys33 linkages. Tissue specificity of these linkages was examined in mice fully labeled by heavy stable isotopes (i.e. SILAC mice). Moreover, we profiled the Ub linkages in brain tissues from patients of Alzheimer disease with or without concurrent Lewy body disease as well as three cellular models of proteolytic stress: proteasome deficiency, lysosome deficiency, and heat shock. The data support that polyUb chains linked through Lys6, Lys11, Lys27, Lys29, and Lys48 mediate proteasomal degradation, whereas Lys63 chains are preferentially involved in the lysosomal pathway. Mixed linkages, including Lys48, may also contribute to lysosomal targeting, as both Lys63 and Lys48 linkages are colocalized in LC3-labeled autophagosomes. Interestingly, heat shock treatment augments Lys11, Lys48, and Lys63 but not Lys29 linkages, and this unique pattern is similar to that in the profiled neurodegenerative cases. We conclude that different polyUb linkages play distinct roles under the three proteolytic stress conditions, and protein folding capacity in the heat shock responsive pathway might be more affected in Alzheimer disease.

Posttranslational modification by monoUb3 or polyUb chains regulates a variety of cellular processes in eukaryotic cells (1, 2). The specificity of Ub signaling in these processes is mediated by E3 ligase-substrate interaction, the recognition of diverse Ub chains by Ub receptors (3), and selective disassembly of Ub chains by deubiquitinating enzymes (4). Recent developments in MS have greatly enhanced the analysis capacity of protein ubiquitination, leading to unexpected findings that any of eight amine groups (on the N terminus and seven Lys residues) in the Ub sequence can be modified by additional Ub moieties rapidly directing substrates to the 26 S proteasome for degradation (9, 10). A single mutation of Lys48 (11) or sexptide mutation of all non-Lys48 Lys sites in Ub (7) results in cellular lethality, indicating that Lys48 chains are essential but not sufficient to maintain cell viability. Unconventional polyUb linkages formed on the N-terminal half of Ub (Lys6, Lys11, Lys27, Lys29, and Lys33) are less characterized but may also contribute to proteasomal targeting (7, 12, 13). For example, Lys11 chains play a role in endoplasmic reticulum associated degradation in yeast (7) as well as in cell cycle regulation (14–16). However, Lys63 linkages and monoUb modification are mainly viewed as a nondegradation signal for protein sorting (17), DNA repair (18), and inflammation (19). Finally, linear polyUb chains are formed via the Ub N-terminal α amino group, but their role in proteolysis is controversial (6, 20, 21). Considering a potentially large number of proteins that are modified by different polyUb linkages, we are still in the early stage of studying the function of these linkages in cellular events and in disease development.

Because Ub-positive inclusions have been long identified in a number of neurodegenerative diseases, and genetic mutations in a few E3 enzyme genes (e.g. parkin) have been identified in familial cases, aberrant Ub signaling is proposed to participate in pathogenesis (22, 23). Proteolytic stress in neurodegeneration may be induced by an imbalance in the protein homeostasis network, including proteins assisting in protein folding, as well as the Ub-proteasome system and autophagy-lysosome degradation. Indeed, enhancement of protein folding capacity in neurodegenerative models of Drosophila or Caenorhabditis elegans attenuates neuronal toxicity (24), and defects in either the proteasomal or lysosomal degradation pathways in mice result in the formation of protein inclusions and some other neurodegenerative phenotypes (25, 26). Thus, the involvement of ubiquitin in protein homeostasis prompted us to systematically investigate different polyUb chains in neurodegeneration.
PolyUb Profiles in Proteolytic Stress Models

Here, we present a refined MS approach to detect major polyUb linkages directly from total cell lysate without pre-enrichment. We have used this approach to measure the level of these linkages in Alzheimer disease tissues and in cellular models of proteolytic stress. The analysis reveals that polyUb chains in mouse and human are primarily composed of Lys48, Lys63, Lys29, and Lys11 linkages. Moreover, the profiled polyUb patterns in the neurodegenerative disease and in models of altered protein homeostasis capacity suggest the differential regulatory functions of these linkages and possible etiology of Alzheimer disease.

EXPERIMENTAL PROCEDURES

Reagents—Reagents used in this study included antibodies against actin (catalog no. sc-1615, Santa Cruz Biotechnology, Santa Cruz, CA), ubiquitin (catalog no. mAb1510, Chemicon Millipore, Billerica, MA), Lys63 polyUb monoclonal Ab (HWA4C4, ebioscience, San Diego, CA); LC3 (catalog no. NB100-2331, Novus Biologicals, Littleton, CO); humanized anti-Lys48 and anti-Lys63 antibodies (27) (a kind gift of V. Dixit); MG-132 and epoxomicin (Boston Biochem., Cambridge, MA); rifuzole (Tocris, Ellisville, MO); and 6-aminonicotinamide and bafilomycin A1 (Sigma).

Mouse and Human Tissues—Mouse chow with heavy stable isotope-labeled lysine (13C6, Cambridge Isotope Labs, Cambridge, MA) was used to feed wild type mice (CBAxC57BL/6) (Lonza, Allendale, NJ) with 10% fetal bovine serum (Invitrogen) modified Eagle’s medium plus 4.5 g/liter glucose and glutamine (Zeiss, Thornwood, NY). For volume determination of autophagosomes (LC3-positive structures), diameter was estimated as the average of measured height and width for each structure using LSM image examiner software and averaged for 10 such structures. Z-axis depth was adjusted to maximize the diameter of most nuclei.

Protein Extraction from Tissues and Cells—Tissue lysate was prepared via homogenization in urea/SDS buffer (10 mM Tris, pH 7.4, 8 m urea, 2% SDS, 4% glycerol, 150 mM NaCl, 5 mM EDTA, 10 mM iodoacetamide) with protease inhibitor mixture (Roche Diagnostics), with 20 strokes in a Dounce tissue grinder with loose pestle, and then 20 strokes with tight pestle (Kontes Glass, Vineland, NJ), followed by cycles of sonication for a 2-s pulse and 30-s cooling on ice, until DNA shearing was apparent by a loss of viscosity. Likewise, total cell lysate of HEK293 and cultured neurons was prepared in the urea/SDS buffer but without homogenization.

For differential protein extraction from HEK293 cells after heat shock response, the cells were lysed with modified radio-immune precipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 mM iodoacetamide, and protease inhibitor mixture). Detergent insoluble pellet was obtained by centrifugation at 20,800 × g for 10 min and was solubilized in 50 µl of urea/SDS buffer. All protein concentrations were determined by bicinchoninic acid assay (Pierce) in the absence of reducing agents.

Targeted MS Measurement by LC-SRM—The quantification of polyUb linkages and selected proteins (E1 and Rpn2) was performed by MS using stable isotope-labeled internal standards, following a previously reported protocol (13, 31). The internal standards included labeled, synthetic GG-linked Ub peptides for absolute quantification (7), and metabolically labeled HEK293 cells (Lys + 8.0142 Da and Arg +10.0083 Da) or mouse tissues (Lys +6.0201 Da) for relative quantification. Labeled cell lysate (20 µg) was prepared in the urea/SDS buffer, spiked into unlabeled lysates mostly at a 1:1 ratio, and resolved on a one-dimensional SDS gel. The gel region above 80 kDa containing the vast majority of polyUb species was used for in-gel trypsin digestion unless indicated differently. This produced pairs of light and heavy peptides. When synthetic peptides were used, they were spiked into gel pieces prior to digestion. Finally, digested peptide pairs were separated by reverse phase LC followed by MS, in which peptide ion pairs of interest were selected for fragmentation and quantified by intensity ratio of coeluting, related product ion pairs, a process termed selected reaction monitoring (SRM) or multiple reaction monitoring. The LC-SRM analysis was performed on a hybrid LTQ-Orbitrap mass spectrometer (Thermo Finnigan, San Jose, CA). The optimized parameters were included in the supplemental materials (see supplemental Table S2).

Fluorescent Protease Activity Assays—The assays were performed using caspase-3/7 substrates (Suc-LVY-AMC or Suc-DEVD-AMC, respectively) with fluorescence activated by proteolysis (Anaspec, Fremont, CA) essentially as described (32). The 100-µl reactions in 96-well plates included 50 µl of total cell lysate (12.5 µg) in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA) and 50 µl of 100 mM substrate (in the lysis buffer plus 2 mM DTT) at 37°C for up to...
fluorescence measurements were taken every 3 min to calculate protease activity.

RESULTS

Improved MS Strategy for Measurement of Ub-Ub Linkages in Mammalian Cells and Tissues—We employed an MS-based approach to measure the level of polyUb linkages, which is represented by the signature Gly-Gly-tagged peptides generated by trypsin digestion of ubiquitinated proteins (31, 33). A key step in this method is the addition of heavy isotope-labeled proteins/peptides (at a known amount) as internal standards for quantifying an unknown amount of unlabeled (light) Gly-Gly-tagged peptides (Fig. 1A). The internal standard proteins/peptides have identical chemical properties to the corresponding native peptides, but they are distinguished by MS to achieve quantitation. Moreover, the method can be used for measuring the abundance of any protein/peptide as long as its labeled internal standard is available. We have used the method with success in yeast (7) and now report its use with mammalian cells and tissues.

We first set out to analyze total Ub in conjugated form and monomeric form (8 kDa) in HEK293 cells. The cell lysate was resolved on an SDS gel that was divided at 12 kDa into two regions for MS analysis (Fig. 1B). The total Ub per 100 μg of lysate was measured to be 32.2 ± 1.1 pmol, and approximately half of ubiquitin was present as free monomer in cells. This result is largely consistent with two reported immunochemical measurements in mammalian cells (34, 35), suggesting the reliability of the newly developed MS approach for ubiquitin quantification.

We continued to measure the abundance of all possible linkages directly from HEK293 cell lysate using the MS strategy. To reduce complexity of the lysate, we decided to analyze proteins in the gel band above 80 kDa, which includes the vast majority of polyUb species (Fig. 1C), similar to the strategy we used for analyzing yeast lysate (7). According to the absolute amount of all eight linkages (totaling 100%), Lys48 (52%) and Lys63 (38%) are the most abundant, followed by Lys29 (8%), then Lys11 (2%) and Lys6 (0.5%) (Fig. 1D-G). Although synthetic, labeled linear (i.e. N-terminal), Lys27 and Lys33 linkage peptides were clearly detected, the native peptides were not identified. According to the detection sensitivity of the method, the maximum contribution of each of the three undetected peptides was <0.5% in the HEK293 cells during the steady growing state without perturbation in protein homeostasis.

To study tissue specificity of the polyUb linkages in mammals, we equally mixed fully labeled SILAC mouse tissues (28) with HEK293 cells and examined the relative level of the four main linkages (Lys48, Lys63, Lys29, and Lys11, Fig. 2). In two generations, the mouse tissues were almost completely labeled (97% labeling efficiency, data not shown). A
Selective Accumulation of Lys^{48}, Lys^{63}, and Lys^{11} Linkages in AD and AD/LBD Cases—To better understand the dysregulation of Ub signaling in neurodegenerative disease, we profiled Ub linkages in postmortem frontal cortex from neurodegenerative cases of Alzheimer disease (AD) without or with neocortical Lewy body Disease pathology (termed AD/LBD cases, supplemental Table S1). The cases were confirmed to show AD and AD/LBD cases.

Coomassie-stained gel of tissue lysates shows different protein patterns. Interestingly, rapidly dividing HEK293 cells have double or more Lys^{11} linkages than the five differentiated tissues, in agreement with a major role of Lys^{11} linkages in mammalian cell division (14). The Lys^{38} linkages and the proteasome subunit Rpn2 are elevated in liver relative to other tissues, suggesting that these Ub chains are important in tissue with a high capacity for protein turnover. Finally, mouse cortex and lung have relatively high Lys^{63} abundance, consistent with endocytic and secretory functions integral to the function of these tissues. Therefore, Ub profiling may provide information about which Ub signaling pathways predominate in a given sample from mammalian cells or tissues.

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Recent studies on autophagy suggest that ubiquitinated species, presumably modified by Lys^{65} linkages, may form inclusions and are then degraded by the autophagy-lysosome system (22, 37). To test whether changes in polyUb occurring at late treatment time points correspond with possible redirection of polyUb conjugates to autophagosomes, we performed immunostaining of MG-132-treated HEK293 cells using recently developed antibodies against Lys^{48} and Lys^{63} linkages (27). Upon 4 h of inhibition, both linkages appeared to be located in inclusions in certain cells, and by 10 h of inhibition, nearly every cell had a large inclusion positive for Lys^{63} and Lys^{48} chains (Fig. 5). The inclusions were further labeled by the autophagosome marker protein LC3. Our data support the idea that prolonged, severe UPS deficiency induces the localization of an increasing
fraction of Lys<sup>63</sup> polyUb-modified substrates into LC3-positive inclusions, suggesting a dynamic flow of proteins between the proteasome and lysosome degradation machineries. In addition, other polyUb linkages (e.g. Lys<sup>48</sup>) may be also involved in this process of directing substrates to the autophagy pathway.

**FIGURE 3.** Lys<sup>11</sup>, Lys<sup>48</sup>, and Lys<sup>63</sup>, but not Lys<sup>29</sup> linkages accumulate in frontal cortex of AD or AD/LBD specimens. A–H, pathological staining of α-synuclein (Lewy body), amyloid β peptide (plaques), Tau (neurofibrillary tangles), and Lys<sup>63</sup>-linked polyUb in control and AD/LBD cases, respectively. Wide-field images were captured with a 10× objective, except a 40× objective was used for D and H. Scale bar is 150 μm for all panels but D and H (20 μm). I, elevated level of Ub in AD/LBD cases in Western blotting. J, analysis of four abundant polyUb linkages in total frontal cortex lysate of the matched control (n = 12), pure AD (n = 12), and AD/LBD cases (n = 12) by LC-MS. The asterisk indicates p value <0.05 according to Student’s t test.

**FIGURE 4.** Proteasome deficiency increases all detected polyUb linkages, but Lys<sup>63</sup> shows a delayed response. A, Western blot of Ub when HEK293 cells were treated with 10 μM epoxomicin. B, Ub linkage profile in a time course during epoxomicin treatment. The signal of Lys<sup>63</sup> linkages was weak compared with others and might be subject to more variation. Rpn2 and E1 were also quantified as two internal loading controls. C, Ub linkage profile in cells treated with 10 μM MG-132. D, PolyUb linkage profile using mouse primary neuronal culture.

PolyUb Profiles in Proteolytic Stress Models

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**JOURNAL OF BIOLOGICAL CHEMISTRY**

10461
PolyUb Profiles in Proteolytic Stress Models

**FIGURE 5.** Lys\(^{48}\) and Lys\(^{63}\) linkages accumulate in inclusions (or inclusion-like structures) after proteasome inhibition. A, Lys\(^{48}\) polyUb was imaged by immunofluorescent staining at 0, 4, and 10 h of MG-132 treatment (10 \(\mu\)M). B, Lys\(^{63}\) polyUb (green) was analyzed together with LC3 (red) under the same conditions. The scale bars represent a distance of 10 \(\mu\)m.

**FIGURE 6.** Lysosomal deficiency increases Lys\(^{63}\) linkages and other polyUb linkages. A, Ub linkage profile of bafilomycin A1-treated (200 nM) HEK293 cells, shown as mean and S.E. Statistical significance (asterisk) was assessed by Student’s t test (\(p < 0.05\)). B, Ub linkage profile of bafilomycin A1-treated primary neurons in a similar time course. C, Immunofluorescence of Lys\(^{48}\), Lys\(^{63}\) polyUb chains, and LC3 with varying bafilomycin A1 (200 nM) treatment time. The nuclei are shown by DAPI staining. Lysosome inhibition appeared to increase the size of LC3/Lys\(^{63}\)/Lys\(^{48}\)-positive structures in HEK293 cells over time. The scale bars represent a distance of 10 \(\mu\)m.

linkages (Lys\(^{11}\), Lys\(^{29}\), and Lys\(^{48}\)) also increased modestly (~1.5-fold). The result suggests that Lys\(^{63}\) linkages are the main polyUb chains responsive to the autophagy inhibition in the HEK293 cell model, whereas other linkages may contribute to this event.

We further attempted the autophagy inhibition treatment in cultured primary embryonic mouse neurons (Fig. 6B), which are thought to support a higher volume of membrane traffic compared with HEK293 cells (Fig. 2). Direct effects of bafilomycin A1 treatment at 4 h are generally consistent with HEK293 trends and include a significant increase in Lys\(^{48}\) and Lys\(^{63}\). After a 10-h treatment, however, conjugated ubiquitin chains decreased to the level at the 0-h time point, suggesting that unlike HEK293 cells, primary neurons cannot maintain ATP-dependent steady state Ub conjugation at 10 h under the bafilomycin A1 concentration of 0.2 \(\mu\)M. Indeed, it was reported that even a 20-fold reduction of this dose may be toxic to neuronal cells treated for longer periods (39).

We further visualized the distribution of Lys\(^{48}\) and Lys\(^{63}\) linkages and LC3 in bafilomycin A1-treated HEK293 cells by triple immunostaining. We noted subtle basal colocalization of Lys\(^{63}\) with LC3-positive structures and more extensive colocalization at 4 and 10 h after bafilomycin A1 treatment, whereas a small fraction of Lys\(^{48}\) linkages were also located in autophagosomes (Fig. 6C). Furthermore the average volume of these stained structures was found to increase from 7.1 \(\mu\)m\(^3\) (0 h) to 8.7 \(\mu\)m\(^3\) (4 h), then to 10.4 \(\mu\)m\(^3\) (10 h) during bafilomycin A1 treatment. In sum, the data suggest that mixed linkages (Lys\(^{63}\) and Lys\(^{48}\)) are located within autophagosomes in response to a block in lysosomal targeting in HEK293 cells, whereas Lys\(^{63}\) chains may play a major role in mediating the sorting of Ub conjugates to autophagosomes.

Heat Shock Induces PolyUb Substrate Accumulation with Linkage Specificity Similar to AD Samples—The third model of proteolytic stress we considered was a comparison of basal versus expanded protein folding capacity. We examined the level of different polyUb linkages in response to heat shock at 42 °C, and concomitant chemical potentiation of the heat shock response by the drug riluzole or the small molecule 6-aminonicotinamide (6-AN). Both chemicals can robustly induce the heat shock response via riluzole-dependent stabilization of HSF1 (40), or possibly through 6-AN inhibition of glucose 6-phosphate dehydrogenase (41). Combinatorial absence or presence of either compound and/or heat shock was tested through a total of six conditions (Fig. 7A). We first assessed protein aggregation by differential extraction, the overall partitioning of total HEK293 protein into radioimmune precipitating assay soluble and insoluble fractions. Heat shock treatment doubled bulk protein insolubility from 5.5 to 11.1%, regardless of pretreatment with either riluzole or 6-AN (Fig. 7B), consistent with the notion that heat shock enhances protein unfolding and aggregation.

We proceeded to profile polyUb linkages in the cell lysate, and found that Lys\(^{11}\), Lys\(^{48}\), and Lys\(^{63}\) linkages were selectively elevated due to heat shock, and this effect was partially attenuated by pretreatment with either riluzole or 6-AN (Fig. 7C). Surprisingly, Lys\(^{29}\) linkages did not change under heat shock, suggesting that the global level of Lys\(^{29}\) was not significantly influenced by heat shock induction. Also in neuronal culture, heat shock treatment augments the level of Lys\(^{11}\), Lys\(^{48}\), and Lys\(^{63}\) linkages but not Lys\(^{29}\) linkages (supplemental Table S3). These results indicate that heat shock results in a unique polyUb linkage pattern, which is more similar to that of AD cases than that associated with the inhibition of proteasome or autophagy pathways.
DISCUSSION

We have developed an MS method for directly measuring polyUb linkages in mammalian cells and tissue and applied this approach toward identifying perturbations in polyUb conjugates in AD. Compared with methods of previous studies in which Ub conjugates were affinity-isolated using Ub binding domains (42) or tagged ubiquitin (43) and then analyzed by MS, this simplified method bypasses the requirement of pre-enrichment of Ub conjugates and thus improves the recovery of Ub conjugates and the sensitivity of detection as well as the analysis turnover time that allows rapid processing of a large number of samples (e.g. 150 samples in this study). This method also reduces variation in measurements of polyUb linkages because pre-enrichment may produce different yields for diverse polyUb chains. Although either heavy stable isotope labeled peptides or proteins can be used as internal standards, the protein standards eliminate error due to variable trypsin digestion efficiency and therefore offer more precise measurement than the peptide standards (7).

In addition to ubiquitin, two Ub-like proteins (Nedd8 and ISG15) also generate GG-tagged peptides on substrates, and thus, our LC-SRM analysis could be confounded. To test this possibility, we analyzed the peptide mixture from the region above 80 kDa on an SDS gel using both shotgun LC-MS/MS and targeted LC-MS/MS. In the targeted analysis, four LC-MS/MS-compatible, “proteotypic” peptides (44) from each of the three proteins (ubiquitin, Nedd8, and ISG15) were selected for MS/MS analysis regardless of their detection by survey MS. This procedure improved the chance to analyze these proteotypic peptides. In both analyses, ISG15 was not identified, and Nedd8 showed a much lower level than ubiquitin (supplemental Table S4). The low amount of Nedd8 relative to ubiquitin may be attributed to modification of cullin proteins by Nedd8 (45), and indeed, four cullin proteins were detected in the shotgun analysis (supplemental Table S4). A critical question is whether Nedd8 could directly modify ubiquitin. Such putative Nedd8-Ub conjugates were not detected in multiple large-scale studies of the ubiquitinated proteome (5, 43, 46), and only trace levels of ubiquitin were found in a study of the Nedd8-associated proteome (45). Therefore, it is unlikely that the eight GG-tagged signature Ub peptides are derived from Nedd8 or ISG15 modification.

The amounts as percentages of polyUb linkages measured in HEK293 cells are slightly different from percentages in yeast: Lys6 (11%), Lys11 (28%), Lys27 (9%), Lys29 (3%), Lys33 (3%), Lys48 (29%), and Lys63 (16%) (7). The mammalian cells have a higher level of Lys48 and Lys63 linkages but a much lower level of Lys6 and Lys11 chains than yeast. The discrepancy could be explained by the species differences in enzymes of Ub conjugation and deubiquitination and relative abundance of chain-specific substrates. It is also possible that the difference could be due to (i) tagging of ubiquitin in yeast and (ii) the analysis procedure, as the yeast analysis was based on affinity captured His-Ub conjugates instead of total cell lysate (7). Our previous study indicated that the effect of ubiquitin tag on the global distribution of polyUb chain linkages is small, less than the effect of genetic background in yeast (7). To test the variation caused by affinity capture, we further compared four abundant linkages (Lys6, Lys11, Lys48, and Lys63) between isolated yeast Ub conjugates and yeast total lysate and found <30% change in any of the linkages (supplemental Fig. S3), suggesting that nickel affinity purification did not have strong bias toward particular chain linkages. Together, the results support that a species difference is the most likely factor underlying the quanti-
PolyUb Profiles in Proteolytic Stress Models

tative inconsistency of polyUb linkages between yeast and mammals. Regardless, it should be noted that the total percentage of different linkages cannot predict their functional significance, as the level of chain-specific individual proteins is not known, and critical regulatory proteins are usually of low abundance.

Whether Ub signaling processes may underlie the etiology of Alzheimer disease has remained an open question. Complex polyUb chains indeed have been implicated in neurodegeneration in previous reports. For instance, Lys\textsuperscript{11}, Lys\textsuperscript{48}, and Lys\textsuperscript{63} linkages are accumulated in affected brain regions in Huntington disease (42), whereas polyUb chains linked through Lys\textsuperscript{6}, Lys\textsuperscript{11}, and Lys\textsuperscript{48} were observed in Tau aggregates isolated from human Alzheimer disease tissue (47), and immunostaining identified Lys\textsuperscript{63} chains associated with tangle of pathology of AD cases (48). Our study gave a more systematic survey of polyUb chains and identified the global augmentation of Lys\textsuperscript{11}, Lys\textsuperscript{48}, and Lys\textsuperscript{63}, but not Lys\textsuperscript{29} chains in total tissue lysate of AD with or without Lewy body disease. Further analysis suggested that the increase in Lys\textsuperscript{11}, Lys\textsuperscript{48}, and Lys\textsuperscript{63} linkages was also correlated with AD disease stage (data not shown).

To recapitulate the unique polyUb pattern in disease, we proceeded to profile polyUb chains in HEK293 cells and cultured neurons, modeling three distinct causes of failed protein homeostasis: proteasomal inhibition, lysosomal deficiency, and heat shock treatment. It was an unexpected finding that the disease-related polyUb signature is best mimicked in cells subjected to heat shock that forces the cells beyond the limits of their protein folding capacity, suggesting that heat shock-responsive mechanisms (e.g. protein misfolding) may be involved in pathogenesis, but roles for the Ub-proteasome system and lysosomal degradation cannot be excluded. Consistent with the data, the Ub ligase ChIP that functions in alleviating protein unfolding stress, has been shown to synthesize polyubiquitin chains on Hsp70 and Hsp90 by Lys\textsuperscript{6}, Lys\textsuperscript{11}, Lys\textsuperscript{48}, and Lys\textsuperscript{63}, but not Lys\textsuperscript{29} (49), although the underlying molecular mechanism for this polyUb pattern needs further investigation.

In addition, detailed time course analysis of polyUb linkages in the cellular models supports a dynamic flow of protein degradation between proteasomal and lysosomal systems. To some extent, accumulation of substrates marked by polyUb for delivery to one system may be redirected to the other system to accommodate the burden of proteolysis. The accumulation of polyubiquitinated proteins is consistent with a concomitant loss of function in the UPS or autophagy. During proteasomal inhibition, Lys\textsuperscript{6}, Lys\textsuperscript{11}, Lys\textsuperscript{27}, Lys\textsuperscript{29}, and Lys\textsuperscript{48} (but not Lys\textsuperscript{63}) linkages increased instantly and the trend continued for 24 h. This result mirrors what was observed in yeast (7), supporting the idea that all non-Lys\textsuperscript{63} linkages function in proteasomal targeting. Interestingly, at later time points (10 h and after), the level of Lys\textsuperscript{63} also increased, suggesting the activation of other, late response pathways. It is highly possible that Lys\textsuperscript{63}-linked substrates are targeted through Ub binding proteins (e.g. p62 and NBR1) to autophagy lysosomal degradation (37, 50), corroborated by immunocytochemistry in which Lys\textsuperscript{63} chains and the autophagy marker LC3 increasingly colocalize after long term inhibition. When the autophagy pathway was inhibited in cells, polyUb chains were changed in an almost reverse pattern: Lys\textsuperscript{63} increased immediately, and other linkages responded later. Interestingly, the LC3-positive autophagosomes contained both Lys\textsuperscript{63}- and Lys\textsuperscript{48}-linked polyUb chains, implying that Lys\textsuperscript{48}-linked substrates are redirected to the lysosomal pathway. In addition to Lys\textsuperscript{63} linkages, Lys\textsuperscript{48} and other non-Lys\textsuperscript{63} linkages may also act as positive signals in this process. Mixed linkages have also been shown to associate with TDP-43 inclusions in a cellular model of frontotemporal lobar degeneration (51). On the other hand, it is also possible that Lys\textsuperscript{63} chains are the dominant signal in sorting of substrates with mixed linkages, and the role of other linkages is passive.

One key issue in this study is whether the increase of polyUb linkages in AD brains is caused by Ub signaling dysfunction in neurons, glial cells, or both. As the biochemical analyses revealed only average values of Ub linkages in all cells in the brain samples, we carried out double immunostaining analysis of Lys\textsuperscript{48} and Lys\textsuperscript{63} linkages with neuronal and glial markers (NeuN and GFAP proteins, respectively, supplemental Fig. S1). Lys\textsuperscript{48}-positive structures are largely present in neurons and are reminiscent of Lewy bodies and neurofibrillary tangles, and Lys\textsuperscript{63}-stained pathological structures may also represent dystrophic neurites and tangles (Fig. 3, supplemental Fig. S1) as reported previously (48). Moreover, double staining showed highly limited overlap between Lys\textsuperscript{63} signals and GFAP-positive cells (supplemental Fig. S1). Our results support that dysfunction of neurons contribute to the up-regulation of polyUb chains, but the role of glial cells cannot be ruled out due to a small number of cases used in this pilot study.

In summary, our data indicate a robust specificity of polyUb signals at the global level of the mammalian proteome under different conditions of proteolytic stress, in which a cultured cell model of limited protein refolding capacity under heat shock treatment closely mimics the polyUb profile of late stage AD. It will be of great interest to study different Ub enzymes (e.g. ubiquitin E2s, E3s, and DUBs), substrates and Ub receptors contributing to the unique polyUb patterns under various stress conditions and in various neurodegenerative disorders.

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PolyUb Profiles in Proteolytic Stress Models

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