Involvement of Polyubiquitin Chains *via* Specific Chain Linkages in Stress Response in Mammalian Cells

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Polyubiquitination plays key roles in various proteasome-dependent and independent cellular events. To elucidate roles in stress response of polyubiquitin chains formed *via* specific chain linkages in mammalian cells, we established NIH3T3 stable cell lines that are capable of conditionally expressing K29R, K48R and K63R ubiquitin mutants, in which the Lys29, Lys48 and Lys63 residues of ubiquitin had been changed to Arg, and we examined the effects of various stresses on their cell viabilities. The expression of K63R ubiquitin mutant decreased viability of the cells post-exposed to ethanol, H₂O₂ and methyl methanesulfonate (MMS), while that of K48R mutant decreased viability of the cells post-exposed to heat shock as well as ethanol, H₂O₂ and MMS. Thus, these results suggest that polyubiquitin chains formed *via* specific chain linkages are involved in the respective stress responses in mammalian cells.

Key words ubiquitin; polyubiquitin chain; stress response; heat shock

In response to various types of stress, organisms ranging from bacteria to humans induce the expression of a small set of universally conserved genes encoding heat shock proteins. Heat shock proteins play key roles not only in the stress response but also in the normal physiological response without stress.

Ubiquitin, a heat shock protein comprising 76 amino acid residues, is highly conserved and widely distributed in eukaryotes.¹⁾ The ubiquitin gene contains a heat shock promoter and its expression is induced by heat shock. Ubiquitin has important roles in post-translational modifications involved in various cellular events, including cell cycle progression, signal transduction, transcription, and DNA repair.^{2–4)} Ubiquitination requires sequential actions of ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3. Ubiquitin is first activated by the formation of a thioester linkage with E1. The ubiquitin is then transferred to one of the E2s *via* a thioester linkage. Subsequently, ubiquitin is transferred to target proteins directly or after reaction with one of the E3s. Multiple ubiquitin molecules are linked to each other and a polyubiquitin chain is formed. Various combinations of E2 and E3 enzymes determine the specificity toward target proteins and also the ubiquitin-chain linkage types of polyubiquitin chains.^{2–4)}

When cells are exposed to chemical, oxidative and heat shock stresses, aberrant forms of stress-sensitive proteins are produced. These abnormal proteins are selectively polyubiquitinated and subsequently degraded by the 26S proteasome. The polyubiquitin chain linked through Lys48 (Lys48-linked chain) functions as a signal for degradation of these abnormal proteins by the 26S proteasome. On the other hand, Lys63-linked polyubiquitin chain formation is required for DNA damage response, independent of proteasome.

We previously reported that polyubiquitinated proteins were accumulated in heat-shocked cells⁵⁾ as revealed by Western blotting with monoclonal antibodies specific for the polyubiquitin chain.⁶⁾ In the present study, we established NIH3T3 stable cell lines that are capable of conditionally expressing K29R, K48R and K63R ubiquitin mutants, in which

the Lys29, Lys48 and Lys63 residues of ubiquitin had been changed to Arg. We investigated whether overexpression of a ubiquitin mutant has an effect on stress response through inhibiting formation of polyubiquitin chains *via* a specific chain linkage. We found that polyubiquitin chains *via* specific chain linkages are involved in various stress responses in mammalian cells.

MATERIALS AND METHODS

Materials Lipofectamine, geneticin (G418), and reverse transcriptase (Superscript II) were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and hygromycin B were obtained from Wako Pure Chemical Ind.

Plasmid Construction Preparation of total RNA from HeLa cells and cDNA synthesis using Superscript II reverse transcriptase from total RNA were preformed by the method described previously.⁷⁾ Ubiquitin DNA was amplified by PCR from cDNA using the following combinations of forward and reverse primers, at the ends of which NotI and NotI sites, respectively, were included: forward primer (5'-GCGGCCG-CATGCAGATCTTCGTGAAGA-3') and reverse primer (5'-GCGGCCGCTTACCCACCTCTGAGACGGAG-3'). The amplified ubiquitin gene was cloned into the LacSwitch system expression vector (pOPRSVI) and analyzed by an ABI 373A DNA sequencing apparatus (Applied Biosystems). For K29R, K48R or K63R ubiquitin mutant in pOPRSVI, the substitution of Lys to Arg was performed according to the methods described by Fukuda *et al.*⁸⁾

Establishment of Stable Cell Lines NIH3T3 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Sigma) supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), and 5% calf serum under a humidified atmosphere in 5% CO₂. The LacSwitch II Inducible Mammalian Expression System (Stratagene) was used to establish stable NIH3T3 cells in which conditional over-expression of ubiquitin and its mutant genes can be achieved. Cells were co-transfected with lipofectamine together with the *lac* re-

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Fig. 1. Inducible Expression of Ubiquitin Mutants and Accumulation of Polyubiquitinated Proteins in NIH3T3 Cells

NIH3T3 cells containing the wild-type (WT), K29R, K48R or K63R ubiquitin plasmid or mock plasmid were incubated in the presence (+) or absence (-) of 5 mM IPTG for 12 h. (A) Total RNA was prepared from the cells, and RT-PCR products prepared were analyzed by agarose gel electrophoresis. (B) The extracts prepared from the cells were subjected to Western blotting with FK2 antibody.

pressor-expressing plasmid P3'C/TMP/equSS and the *lac* repressor-repressible pORSVI plasmid expressing ubiquitin or its mutant. Co-transfected cells were selected in the presence of hygromycin B (100 μ g/ml) and G418 (120 μ g/ml) and cloned by a cloning cap. Cloned cells were screened for inducible mRNA expression by RT-PCR. To overcome repression by *lac* repressor and consequently express ubiquitin or its mutant, isopropyl- β -D-galactopyranoside (IPTG) (5 mM) was added to the medium and cells were further incubated for 12 h. After preparation of total RNA from each clone and reverse transcription, exogenous ubiquitin or its mutant cDNA was amplified by PCR using the following forward and reverse primers: To avoid amplification of endogenous ubiquitin cDNA, the reverse primer (5'-CGGGTTC-CTTCCGGTATTG-3') was designed for priming on the DNA sequence of thymidine kinase polyA additional signal in the pORSVI vector and the forward primer (5'-GCGGC-CGCATGCAGATCTTCGTGAAGA-3') was designed for priming on the N-terminus of ubiquitin. Amplified products were analyzed by agarose gel electrophoresis. The ones showing the highest levels of gene expression were chosen for further analysis.

Stress Response and Western Blotting In experiments on heat shock stress, 2×10^5 of NIH3T3 cells were incubated in a water bath at 43, 44 or 45 $^{\circ}$ C for 30 min and were then incubated at 37 $^{\circ}$ C in a humidified CO₂ incubator for various periods. In experiments on chemical stresses with ethanol, H₂O₂ and methyl methanesulfonate (MMS), the respective reagents were added to the medium and cells were further incubated for indicated periods. For Western blotting, cells were solubilized in SDS-PAGE sample buffer containing 2 mM *N*-ethylmaleimide, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin and 5 μ g/ml aprotinin, boiled for 5 min, and sonicated for 30 s with an immersible tip-type sonicator in order to shear the chromosomal DNA. The lysate was subjected to SDS-PAGE on a 9% polyacrylamide gel followed by Western blot analysis using FK2 antibody.⁶ Detection was performed using an enhanced chemiluminescence system (Amersham), and the bands were visualized with X-ray film.

Cell Viability Assay Cloned stable cells were cultured at 37 $^{\circ}$ C in 3 ml of medium containing 5 mM IPTG for 12 h to induce gene expression. The cells were exposed to heat shock or chemical stress, further incubated at 37 $^{\circ}$ C, and then subjected to a cell viability assay by the MTT method⁹ under

a Nikon TMD fluorescence microscope. Phase-contrast photographs were taken (magnification, 200).

RESULTS AND DISCUSSION

In order to address the question of whether polyubiquitin chains formed *via* specific chain linkages could play characteristic roles in various stress responses in mammalian cells, we established NIH3T3 stable cell lines that are capable of expressing the wild-type ubiquitin and its K29R, K48R and K63R mutants, induced by IPTG treatment. It is possible that inhibition of the formation of polyubiquitin chains *via* specific ubiquitin-chain linkages affects cell viability. The Lac-Switch II Inducible Mammalian Expression System containing the *lac* repressor and *lac* repressor-repressible ubiquitin plasmids was used to establish the stable cell lines for conditional gene expression. Several independent clones showing inducible expression of the wild-type or mutant ubiquitin were obtained, and the ones showing the highest levels of gene expression were subjected to RT-PCR (Fig. 1A). It was found that expressions of the wild-type ubiquitin and its three mutants can be induced by treatment with IPTG. To analyze changes in the protein levels of ubiquitin conjugates in the established stable clones, the extracts of cells that had been treated with IPTG were subjected to Western blotting with FK2 antibody (Fig. 1B). Unexpectedly, little difference between the wild-type and each of the three mutant cells was observed, possibly because ubiquitin conjugates are thought to be formed mainly *via* endogenous ubiquitin.

To determine specific roles of Lys29-, Lys48- and Lys63-linked polyubiquitin chains in recovery from heat shock and chemical stresses, we examined the effects of heat shock and treatments with ethanol, H₂O₂ and MMS on viability of the stable cell line expressing ubiquitin or its mutant, in which formation of a Lys29-, Lys48- or Lys63-linked polyubiquitin chain could be blocked (Fig. 2). In expression of the wild-type (WT) ubiquitin or control plasmid (mock), little change in cell viability was observed in any stress treatment. On the other hand, the viability of K48R mutant-expressing cells in response to heat shock was significantly decreased compared with the viability of cells expressing the wild-type and K29R and K63R mutants (Fig. 2A). Expression of the K63R mutant caused loss of cell viability in response to ethanol, H₂O₂ and MMS treatments, as was the case with K48R mutant expression, (Figs. 2B, C, D). In expression of the K29R mutant,

Fig. 2. Effects of Expression of Ubiquitin Mutants on Viability of NIH3T3 Cells Post-Exposed to Various Stresses

NIH3T3 cells expressing the wild-type (WT, closed circles), K29R (open squares), K48R (closed squares), or K63R (open triangles) ubiquitin were treated at 45 °C for 15 or 30 min (A) and with ethanol (B), H_2O_2 (C) and MMS (D) for 2 h at indicated concentrations. In (A), after heat shock treatment, cells were returned to the incubator at 37 °C followed by cultivation for 24 h: The value just before heat shock treatment was defined as 100%. Mock expression is indicated by open circles. Data are mean $\pm$ S.E.M. (bars) values of six experiments.

a slight decrease in cell viability was observed in response to ethanol and H_2O_2 (Figs. 2B, C) but not in response to heat shock and MMS (Figs. 2A, D). Although a certain combination of E2–E3 catalyzes formation of Lys29-linked polyubiquitin chain *in vitro*,¹⁰ the role of Lys29-linked chain *in vivo*, including its role in stress response, remains unclear. Thus, these findings suggest that formation of both Lys48- and Lys63-linked polyubiquitin chains is essential for stress response to ethanol, oxidative stress and DNA damage, the former chain formation being essential for heat shock stress.

Next, we observed cell morphology of mutant ubiquitin-expressing cells post-exposed to heat shock (Fig. 3). Expression of the K48R mutant caused remarkable damage of cells and cellular morphological change. In contrast, the wild-type and other mutant-expressing cells showed thermotolerance. These results are consistent with the results shown in Fig. 2A. Thus, it can be inferred that the formation of a Lys48-linked polyubiquitin chain is essential for acquisition of thermotolerance, implying that misfolded and denatured proteins produced by heat treatment are selectively modified with the K48-linked polyubiquitin chains and degraded by the 26S proteasome.

Involvement of Lys63-linked polyubiquitin modification in DNA-damage response was first proposed in budding yeast¹¹ and has been extensively investigated in this eukaryote. The DNA damage can block the progress of DNA polymerase. During post-replicative repair, proliferating cell nuclear antigen (PCNA), a processivity factor essential for repair, is monoubiquitinated through Rad6 (a member of the E2 family, Ubc2) and Rad18 (a RING finger domain-containing E3), which leads to translesion DNA synthesis; Subsequently, PCNA is polyubiquitinated *via* Lys63 linkage by Mms2/Ubc13 ubiquitin conjugating enzyme complex and Rad5 ubiquitin ligase, which promotes error-free bypass of DNA lesions.^{12–14} In addition, Rad18 is mono- and polyubiquitinated in a Rad6-dependent manner: Monoubiquitination of Rad18 is induced through self-association, resulting in its cytoplasmic localization, while polyubiquitination of

Fig. 3. Effects of Expression of Ubiquitin Mutants on Morphology of NIH3T3 Cells Post-Exposed to Heat Shock

After heat shock treatment at 37, 43 or 45 °C for 30 min, the cells were incubated at 37 °C for 24 h and observed under a microscope. Phase-contrast photographs were taken (magnification, 200 $\times$).

Rad18 leads to its degradation by the proteasome.¹⁵ Our finding in mammalian cells that the expression of K63R and K48R ubiquitin mutants decreased viability of the cells post-exposed to DNA damage induced by MMS is consistent with the above-described results obtained in yeasts. Identification of ubiquitin linkage types of polyubiquitin chains formed in response to various stresses by a proteomics approach and/or by using a monoclonal antibody to a specific ubiquitin linkage type is necessary to clarify the roles of the specific polyubiquitin chains in the respective stress responses.

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