Keap1 degradation by autophagy for the maintenance of redox homeostasis

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The Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) system is essential for cytoprotection against oxidative and electrophilic insults. Under unstressed conditions, Keap1 serves as an adaptor for ubiquitin E3 ligase and promotes proteasomal degradation of Nrf2, but Nrf2 is stabilized when Keap1 is inactivated under oxidative/electrophilic stress conditions. Autophagy-deficient mice show aberrant accumulation of p62, a multifunctional scaffold protein, and develop severe liver damage. The p62 accumulation disrupts the Keap1-Nrf2 association and provokes Nrf2 stabilization and accumulation. However, individual contributions of p62 and Nrf2 to the autophagy-deficiency-driven liver pathogenesis have not been clarified. To examine whether Nrf2 caused the liver injury independent of p62, we crossed liver-specific *Atg7::Keap1-Alb* double-mutant mice into *p62*- and *Nrf2*-null backgrounds. Although *Atg7::Keap1-Alb::p62*^{-/-} triple-mutant mice displayed defective autophagy accompanied by the robust accumulation of Nrf2 and severe liver injury, *Atg7::Keap1-Alb::Nrf2*^{-/-} triple-mutant mice did not show any signs of such hepatocellular damage. Importantly, in this study we noticed that Keap1 accumulated in the *Atg7*- or *p62*-deficient mouse livers and the Keap1 level did not change by a proteasome inhibitor, indicating that the Keap1 protein is constitutively degraded through the autophagy pathway. This finding is in clear contrast to the Nrf2 degradation through the proteasome pathway. We also found that treatment of cells with *tert*-butylhydroquinone accelerated the Keap1 degradation. These results thus indicate that Nrf2 accumulation is the dominant cause to provoke the liver damage in the autophagy-deficient mice. The autophagy pathway maintains the integrity of the Keap1-Nrf2 system for the normal liver function by governing the Keap1 turnover.

electrophile | polyubiquitination

The Kelch-like ECH-associated protein 1 (Keap1)-NF-E2-related factor 2 (Nrf2) system regulates the expression of a battery of cytoprotective genes in response to electrophilic and oxidative stresses (1–6). Keap1 serves as a stress sensor and an adaptor component of Cullin 3 (Cul3)-based ubiquitin E3 ligase (7). Under normal (unstressed) conditions, Keap1 constitutively ubiquitinates Nrf2, resulting in the rapid degradation of Nrf2 through the proteasome pathway, thus maintaining a low level of cellular Nrf2. Upon cellular exposure, electrophiles modify reactive cysteine residues of Keap1 and inhibit its activity as an E3 ligase component, provoking Nrf2 stabilization and subsequent accumulation in the nucleus. Accumulated Nrf2 binds to a *cis*-acting antioxidant/electrophile responsive element (8) and activates the transcription of a cluster of genes encoding cytoprotective enzymes (9). After the inactivation of Keap1, one can expect the recovery of Keap1 activity if Keap1 turns over steadily and intact Keap1 is continuously supplied. However, the degradation pathway and turnover profile of Keap1 remains to be elucidated.

Autophagy is known as a bulk protein degradation system observed during nutrient deprivation (10–12). Autophagy also plays

an important role in eliminating proteins and organelles that are damaged because of oxidative stress and aging (13). A170/SQSTM1 (Sequestosome 1)/p62 has emerged as a multifaceted adaptor protein that exhibits diverse biological functions through the interaction with numerous proteins (14). In particular, p62 plays an important role in autophagy because it serves as a substrate carrier. It is known that p62 possesses a microtubule-associated protein 1 light-chain 3 (LC3)-interacting region (LIR) and ubiquitin-associated domain (UBA), and interacts with LC3 on the isolation membrane of autophagosomes and ubiquitinated substrate proteins through LIR and UBA, respectively (15–17). Substrate proteins are sequestered into autophagosomes via polyubiquitin chains, LC3 and p62, and are degraded through the autophagy pathway (17–19).

Autophagy deficiency in the liver because of liver-specific disruption of the *Atg7* gene in mice (*Atg7*^{flax/flax}::*Alb-Cre*; referred to as *Atg7-Alb* in this study) is found to give rise to aberrant accumulation of p62 and subsequent liver damage (16). We also found that p62 directly associates with Keap1 and disrupts the interaction between Keap1 and Nrf2, leading to the stabilization of Nrf2 (20). The liver injury in *Atg7-Alb* mice was rescued by the deletion of either the Nrf2 or p62 gene, but it was deteriorated by the deletion of the Keap1 gene (16, 20). However, individual contributions of Nrf2 and p62 to the liver pathogenesis remain to be clarified.

It was reported that Keap1 is ubiquitinated by a Cul3-dependent complex, although the 26S proteasome does not degrade Keap1 (21). Considering the direct interaction of Keap1 with p62, these results suggest the possibility that p62 interacts with Keap1 and results in Keap1 degradation through the autophagy pathway. Indeed, disruption of the *Atg7* gene in livers generates inclusion bodies containing p62 and Keap1 (20), suggesting the concomitant accumulation of p62 and Keap1 upon the autophagy deficiency. Therefore, in this study we examined contribution of Nrf2 to the liver pathogenesis in autophagy-deficient animals by using conditional *Atg7::Keap1* double-mutant mice because these mice exhibit more severe liver damage than do the *Atg7-Alb* single-mutant mice. We crossed the *Atg7::Keap1* double-mutant mice into the *Nrf2*-null and *p62*-null backgrounds and generated *Atg7::Keap1-Alb::Nrf2*^{-/-} and *Atg7::Keap1-Alb::p62*^{-/-} mice, respectively. We found that the former did not show any signs of liver injury, but the latter displayed severe injury of the liver similar to the *Atg7::Keap1-Alb* mice.

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Taguchi et al.

Fig. 2. Accumulation of p62 in *Atg7::Keap1-Alb::Nrf2*^{-/-} liver. Immunoblot analysis of whole-cell extracts (Whole Cell) and nuclear extracts (Nucleus) of liver (A) and relative mRNA expression measured by quantitative real-time PCR (B) in livers from various genotypes of mice. αTubulin and Lamin B were used as loading controls. Lanes 1 *Atg7*^{flx/flx} (Control), 2 *Atg7*^{flx/flx}-*Alb*, 3 *Atg7*^{flx/flx}::*Keap1*^{flx/flx}-*Alb*, 4 *Atg7*^{flx/flx}::*Keap1*^{flx/flx}-*Alb*::*p62*^{-/-}, and 5 *Atg7*^{flx/flx}::*Keap1*^{flx/flx}-*Alb*::*Nrf2*^{-/-}. Data are the means ± SD. **P* < 0.05, ***P* < 0.01 compared with control.

Nrf2^{-/-} mice were also comparable with that in *Atg7::Keap1-Alb* mice, but the *Nrf2* expression was absent in the former. These results thus demonstrate that in the autophagy-deficient mice *Nrf2* accumulation and activation provokes the liver injury, even in the absence of p62, but mere p62 accumulation in the absence of *Nrf2* does not provoke the liver injury.

Increase of Keap1 Protein Level in *Atg7-Alb* and *p62*^{-/-} Mice. During the course of these analyses, we noticed that Keap1 protein is increased in the *Atg7-Alb* mouse liver (Fig. 2A). Because Keap1 directly interacts with p62 (20), we surmised that Keap1 may be recruited to the isolation membrane by p62 and degraded through autophagy.

To address this hypothesis, we examined the levels of both the Keap1 protein and *Keap1* mRNA in the *Atg7-Alb* and *p62*-null mouse livers. The Keap1 protein level was markedly higher in the *Atg7-Alb* mouse livers than in the control livers (Fig. 3A). The p62 and *Nrf2* protein levels were also higher in the *Atg7-Alb* mouse livers. In contrast, the *Keap1* mRNA levels in the *Atg7-Alb* mouse livers were comparable with that of the control livers (Fig. 3B). Similarly, deletion of the *p62* gene increased the Keap1 protein level (Fig. 3C), but the *Keap1* mRNA level in the *p62*-null liver was comparable with that of the control livers (Fig. 3D). These results indicate that the increase of Keap1 protein in the absence of autophagy or in the absence of p62 is not attributable to the transcriptional activation of the *Keap1* gene, but that enfeebled degradation of Keap1 protein causes the Keap1 accumulation.

Importantly, the *Nrf2* gene disruption reduced the level of *Keap1* mRNA irrespective of the status of the *Atg7* gene (Fig. 3B), whereas the *Nrf2* gene disruption reduced the Keap1 protein level only in the *Atg7*-null background and not in the control background (Fig. 3A). The former result is consistent with the observation that Keap1 is an *Nrf2* target gene (22). We surmise that when the autophagy pathway is intact, the Keap1 protein level does not reflect the *Keap1* mRNA level. However, when autophagy is inhibited, the protein level directly reflects the

mRNA level. These results further support the contention that Keap1 is degraded through autophagy.

Keap1 Degradation in Cultured Cells. We then examined the Keap1 protein level in HepG2 cells when autophagy activity was modulated. Two forms of LC3, LC3-I (slowly migrating band) and LC3-II (rapidly migrating band), and p62 were detected for monitoring the autophagy activity. We first treated HepG2 cells with MG132, a proteasome inhibitor, and found that the Keap1 level hardly changed during 24 h (Fig. 4A), indicating that the proteasome pathway is not involved mainly in the degradation of Keap1. Unexpectedly, p62 and both forms of LC3 were increased upon the MG132 treatment, and the reason for these accumulations was not clear at present.

To induce autophagy we subjected HepG2 cells to nutritional starvation. Supporting the successful induction of autophagy, p62 was undetectable in this nutritional starvation condition. Consistent with our expectation, Keap1 protein level was markedly decreased in HepG2 cells upon nutrient starvation (Fig. 4B). This observation supports the contention that Keap1 is degraded through autophagy.

On the other hand, when we used three kinds of autophagy inhibitors (i.e., torin1, E64/pepstatin A, and bafilomycin), these inhibitors did not increase the Keap1 protein levels (Fig. 4C–E). As these inhibitors increased the levels of p62 and both forms of LC3, the autophagy pathway seemed to be successfully inhibited. The reason why Keap1 protein level does not increase upon exposure to these autophagy inhibitors remains to be elucidated. One plausible explanation is that the production of Keap1 might be inhibited in the presence of these inhibitors.

Fig. 3. Increase of the Keap1 protein level in *Atg7-Alb* and *p62*^{-/-} mice. Protein and mRNA expression levels of Keap1, *Nrf2*, and p62 in *Nrf2*^{-/-}, *Atg7-Alb*, *Atg7-Alb::Nrf2*^{-/-}, and *Keap1-Alb* mice (A and B) and *p62*^{-/-} mice (C and D). Immunoblot analyses were carried out using whole-cell extracts (Whole Cell) and nuclear extracts (Nucleus) of liver (A and C). αTubulin and Lamin B were used as loading controls. *Atg7-Alb* was used as a positive control in C. Data are the means ± SD of three mice. **P* < 0.05, ***P* < 0.01 compared with each control (B).

Fig. 4. Effects of a proteasome inhibitor, starvation and autophagy inhibitors on the protein levels of Keap1 and p62. Immunoblot analyses were carried out using whole-cell extracts of HepG2 cells. Keap1, p62, and LC3 proteins were detected with specific antibodies. α Tubulin was detected as a loading control. A proteasome inhibitor, MG132 (3 μ M) (A), nutritional starvation in HBSS (B), and three kinds of autophagy inhibitors, Torin1 (0.1 μ M) (C), E64/PepstatinA (10 μ g/mL) (D), and Bafilomycin (0.1 μ M) (E) were applied. These experiments were repeated multiple times and found to be reproducible. Representative results are shown.

Keap1 Degradation Is Accelerated in Electrophilic Stress Conditions. Because Keap1 possesses multiple reactive cysteine residues that are directly modified by electrophiles, we are curious whether Keap1 stability is affected by the electrophilic modifications. We measured the half-life of Keap1 in HepG2 cells to be 12.7 h under normal conditions by means of cycloheximide (CHX) treatment [Fig. 5A (Upper Left) and B]. The Keap1 half-life was similarly measured in the presence of electrophiles to determine the fate of Keap1 after reacting with electrophiles. When HepG2 cells were treated with tBHQ plus CHX, the half-life of Keap1 was shortened to 3.4 h [Fig. 5A (Lower Left) and B]. When diethyl maleate (DEM) or 1,2-naphthoquinone (1,2-NQ) was applied instead of tBHQ, the half-life was 10.4 and 7.1 h, respectively [Fig. 5A (Right) and B].

These results indicate that some of electrophiles accelerate the degradation of Keap1 and suggest that Keap1 modified with these electrophiles becomes a preferable target of autophagy and is more readily degraded than unmodified Keap1. Because Nrf2 activates the Keap1 gene expression (22) and Keap1 inactivation by electrophiles stabilizes Nrf2 and activates Nrf2-mediated transcription (23), we surmise that the accelerated turnover of Keap1 contributes to the efficient recovery of the Keap1 activity from the electrophilic modification and inactivation. Thus, these results support the

notion that the autophagy pathway contributes to the maintenance of the Nrf2 activity and cellular redox homeostasis.

Discussion

The autophagy-deficient *Atg7-Alb* mice suffer from liver injury and this can be rescued by the simultaneous deletion of the Nrf2 gene (20). However, because *Atg7-Alb::Nrf2^{-/-}* double-mutant mice display the aberrant accumulation of p62, as is the case for *Atg7-Alb* mice, it has not been clear whether the Nrf2 induction by itself provokes the liver injury in the absence of p62. As summarized in Fig. 6, this study clearly demonstrates that constitutive activation of Nrf2 is the dominant cause of the liver injury in the autophagy-impaired mice. The other pathways influenced by the p62 accumulation, such as the NF- κ B pathway, are suggested not to make substantial contributions to the development of liver injury. Another important finding in this study is that Keap1 is degraded through the autophagy pathway in a p62-dependent manner.

The physical and functional interactions between Keap1, Nrf2, and p62 have been clarified; p62 directly interacts with Keap1 and disrupts the association between Keap1 and Nrf2, resulting in the stabilization and nuclear accumulation of Nrf2 (20). The accumulation of Nrf2 activates transcription of a number of cytoprotective genes. In contrast, the p62 gene is an Nrf2 target, so that Nrf2 induces the production of p62 (24, 25). The combination of these two arms forms a positive feedback loop in the antioxidant response (i.e., Nrf2 induces p62 and p62 induces Nrf2) (26). It has also been reported that p62 determines the basal level of Keap1 and basal activity of Nrf2-dependent transcription through regulation of the Nrf2 protein level (27).

Autophagy has been regarded as a process of bulk protein degradation that occurs during starvation for the redistribution of nutrients (10–12). Since the identification of p62 as a substrate receptor for autophagy, the roles of autophagy plays in selective

Fig. 5. Half-life of Keap1 protein in the presence or absence of electrophilic stimuli. Immunoblot analyses were carried out using whole-cell extracts of HepG2 cells treated with various electrophiles in the presence of CHX (A). Band intensities of Keap1 were quantified, normalized to those of α Tubulin, and plotted (B). Representative results are shown. The half-life of Keap1 under each condition was calculated based on the data in A. Data are the means $\pm$ SD of three independent experiments. The half-life of Keap1 was determined in the presence of DEM (100 μ M), 1,2-NQ (50 μ M), and tBHQ (100 μ M) with or without CHX (0.1 mg/mL).

Fig. 6. Impaired autophagy accumulates Keap1 as well as p62 and leads to Nrf2-dependent liver injury. Autophagy is one of proteolytic systems. When the isolation membrane emerges, LC3 is attached to the membrane and binds p62, which in turn links to ubiquitinated substrate proteins. Autophagosome fuses with lysosome to form autolysosome, resulting in the protein degradation. Autophagy impaired by *Atg7* deletion accumulates Keap1 as well as p62. Constitutive accumulation of Nrf2 is the dominant cause of the liver injury in impaired autophagy independent of p62.

protein degradation have been widely recognized (14). Autophagy-linked FYVE has been suggested to cooperate with p62 in selective autophagy, serving as a scaffold protein for the assembly of p62-positive cytoplasmic bodies and the recruitment of autophagosomal membranes (28). In addition, the neighbor of BRCA1 gene 1 has been identified as an alternative substrate receptor for selective autophagy (29). Furthermore, dishevelled homolog 2 was recently identified as a specific substrate of inducible autophagy under starvation conditions (30). Keap1 is another example of a specific substrate of autophagy. In this regard, it should be noted that Keap1 is degraded in the autophagy pathway even under the basal and native/unmodified conditions.

In contrast to our observations, one report showed that Keap1 facilitated the p62-mediated ubiquitin aggregate clearance via autophagy, but the report did not show that Keap1 itself was degraded through autophagy (31). In our study using mouse livers *in vivo*, the Keap1 protein level increased in the autophagy-deficient *Atg7-Alb* mice compared with the control mice and the decrease of Keap1 protein was accelerated when autophagy was induced under the nutritional starvation. Based on these lines of evidence, we conclude that, at least in hepatocytes, Keap1 is degraded by autophagy in a p62-dependent manner.

One unsolved question is the mechanism that targets Keap1 for autophagy in a p62-dependent manner, especially the precise interaction mode between Keap1 and p62. One plausible explanation is the direct binding of Keap1 and p62, as p62 has been shown to interact directly with the Keap1 DC domain through the Keap1-interacting region (KIR) of p62 (20, 26). However, the formation of an LC3-p62-Keap1 ternary complex on the autophagosome membrane may cause a steric hindrance because the KIR is adjacent to the LC3-interacting region LIR of p62. An alternative explanation is the binding of Keap1 and p62 through the polyubiquitin chain. Because Keap1 ubiquitination has been reported (21), the UBA domain of p62 may associate with the polyubiquitin chain of Keap1.

Polyubiquitination occurs through an isopeptide bond formation between the ϵ -amino moiety of a lysine residue in one ubiquitin molecule and the C-terminal carboxyl moiety of the next ubiquitin molecule. Among the diverse chain linkages that involve seven lysine residues in ubiquitin (32, 33), Lys48- and Lys63-linked chains have been well characterized. Lys48-linked polyubiquitin

chains are the most common signals for proteasomal degradation, whereas Lys63-linked polyubiquitin chains are involved in the other processes, such as protein trafficking and DNA repair (34). Several lines of evidence support the close association between the latter and autophagy. Indeed, our preliminary data indicate the selective hyper-accumulation of proteins possessing Lys63-linked polyubiquitin chains in the livers of the autophagy-deficient *Atg7-Alb* mice (Fig. S1). The accumulation of Lys63-polyubiquitinated proteins are also observed in the brains of *p62*^{-/-} mice (35). Although the UBA domain of p62 binds both of the Lys48- and Lys63-linked polyubiquitin chains in an *in vitro* pull-down analysis, p62 selectively promotes the extension of Lys63-linked polyubiquitin chains *in vivo* through an interaction with the E3 ubiquitin ligase, TNF receptor-associated factor 6 (18). Keap1 is known to be polyubiquitinated but is not degraded via the proteasome (21). Thus, the possibility exists that Keap1 may retain the Lys63-polyubiquitination, which interacts with p62, and that Keap1 is destined for degradation through autophagy.

Materials and Methods

Chemicals. Chemicals were purchased as follows: Bafilomycin A1, HBSS, and tBHQ were from Sigma-Aldrich; MG132, CHX, and DEM were from Wako Chemicals; Torin1 was from Tocris Bioscience; and E64 and Pepstatin A were from the Peptide Institute. 1,2-NQ was kindly provided by Yoshito Kumagai (University of Tsukuba, Ibaraki, Japan). All of the other chemicals used were obtained from commercial sources and were of the highest grade available.

Mice. *Atg7*^{flox/flox} (control), *Nrf2*^{-/-}, *Atg7*^{flox/flox}::*Albumin-Cre* (*Atg7-Alb*), *Atg7*^{flox/flox}::*Albumin-Cre*::*Nrf2*^{-/-} (*Atg7-Alb*::*Nrf2*^{-/-}), *Keap1*^{flox/flox}::*Albumin-Cre* (*Keap1-Alb*), *Atg7*^{flox/flox}::*Keap1*^{flox/flox}::*Albumin-Cre* (*Atg7*::*Keap1-Alb*), *Atg7*^{flox/flox}::*Keap1*^{flox/flox}::*Albumin-Cre*::*p62*^{-/-} (*Atg7*::*Keap1-Alb*::*p62*^{-/-}), *Atg7*^{flox/flox}::*Keap1*^{flox/flox}::*Albumin-Cre*::*Nrf2*^{-/-} (*Atg7*::*Keap1-Alb*::*Nrf2*^{-/-}), and *p62*^{-/-} mice were generated and maintained as described previously (16, 36–38). The mice were given water and rodent chow *ad libitum*. All of the mice were kept under specific pathogen-free conditions and were treated according to the regulations of The Standards for Human Care and Use of Laboratory Animals of Tohoku University and Guidelines for Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Plasma ALT and AST were measured by DRI-CHEM 7000V (Fujifilm). Livers were fixed in Mildform 10N and embedded in paraffin for H&E staining.

Cell Culture. A human hepatocellular carcinoma cell line, HepG2, was grown in DMEM containing 10% (vol/vol) FBS, 100-units/mL penicillin, and 100- μ g/mL streptomycin. The cells were incubated at 37 °C in a humidified incubator under 5% (vol/vol) CO₂. The cells (0.45 $\times$ 10⁶ cells) were plated onto 35-mm cell culture dishes. At 16 h after seeding, the cells were treated with chemicals for the indicated time intervals. For starvation, medium was replaced with HBSS.

Real-Time PCR. Total RNA was isolated using ISOGEN (Nippon Gene), and was transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). Quantitative PCR analysis was carried out using an Applied Biosystems 7300 PCR system, and qPCR Mastermix Plus (Eurogentec) was used to detect *Nqo1* and *p62* transcripts. The data were normalized to ribosomal RNA expression. All primers and probes were used as reported previously (20).

Immunoblot Analysis. Tissues were homogenized in 9 volumes of 0.25 M sucrose, and the 10% (vol/vol) homogenate was filtered through a 100- μ m membrane. Nuclear fraction was prepared as previously reported with some modifications (39). The cells were lysed in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 10% (vol/vol) glycerol, and 2% (wt/vol) SDS]. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology) with BSA as the standard. The samples were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). Anti-Nrf2 (40), anti-Keap1 (41), anti-p62 (Progen; GP62-C), anti-LC3B (Cell Signaling Technology; 27755), anti-Lamin B (Santa Cruz Biotechnology; sc-6217), anti-Ub (P4D1) (Santa Cruz Biotechnology; sc-8017), anti-Ubiquitin Lys48-Specific (Millipore; 05-1307), anti-Ubiquitin Lys63-Specific (Millipore; 05-1308), and anti- α Tubulin (Sigma-Aldrich; T9026) antibodies were used.

Statistical Analysis. All of the differences were analyzed with Student *t* test. *P* < 0.05 was considered statistically significant.

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