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Coordination of oxidized protein hydrolase and the proteasome in the clearance of cytotoxic denatured proteins

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Abstract

Intracellular accumulation of denatured proteins impairs cellular function. The proteasome is recognized as an enzyme responsible for the effective clearance of those cytotoxic denatured proteins. As another enzyme that participates in the destruction of damaged proteins, we have identified oxidized protein hydrolase (OPH) and found that OPH confers cellular resistance to various kinds of oxidative stress. In this study, we demonstrate the roles of the proteasome and OPH in the clearance of denatured proteins. The inhibition of proteasome activity results in the elevation of protein carbonyls in cells under oxidative stress. On the other hand, cells overexpressing OPH retain higher resistance to oxidative stress, even though the proteasome activity is inhibited. Furthermore, upon inhibition of the proteasome activity, OPH is recruited to a novel organelle termed the aggresome where misfolded or denatured proteins are processed. Thus, OPH and the proteasome coordinately contribute to the clearance of cytotoxic denatured proteins.

Keywords: Protein degradation; Misfolded proteins; Denatured proteins; Oxidized protein hydrolase; Proteasome; Aggresome; Oxidative stress

Oxidative stress such as free radicals and other oxidants produced in an inevitable consequence of aerobic metabolism causes continuous formation of oxidatively damaged proteins within cells [1–3]. Therefore, the prevention of accumulation of oxidized cellular proteins is one of the major functions of the proteolytic machinery of mammalian cells.

We have previously demonstrated that oxidized protein hydrolase (OPH), which preferentially degrades oxidatively damaged proteins, confers cellular resistance to prooxidative reagents such as H_2O_2 and paraquat [4]. Indeed, the level of protein carbonyls, a marker of protein oxidation, was significantly lower in COS-7-OPH cells, which stably overexpress OPH, than that in parental COS-7 cells when cells were exposed to oxidative stress [4].

As one of the ultimate machinery that cells use to ensure the quality of intracellular proteins, the proteasome is known to act for the selective destruction of misfolded or damaged proteins [5]. There are series of studies suggesting that the proteasome is responsible for the degradation of oxidized proteins [5,6], although it is still subject to debate whether or not ubiquitination is involved in this process [7,8].

In this study, we evaluate the roles of OPH and the proteasome in protection against oxidatively damaged proteins. A selective proteasome inhibitor, epoxomicin (Epox), augments protein carbonylation induced by paraquat in COS-7 cells but not in COS-7-OPH cells. However, augmentation of paraquat-induced protein carbonylation is observed in COS-7-OPH cells when activities of both OPH and the proteasome have been inhibited. Furthermore, upon inhibition of proteasome activity by Epox, OPH accumulates and, strikingly, becomes concentrated into a single prominent juxtanuclear inclusion body that is termed the aggresome [9]. These

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results suggest that OPH and the proteasome coordinately contribute to the elimination of cytotoxic damaged proteins.

Materials and methods

Materials. *N*-acetyl-L-alanine *p*-nitroanilide (AANA), methyl viologen (paraquat), lactacystin (LC), mouse anti-FLAG monoclonal antibody (M2), and mouse anti-γ-tubulin (GTU-88) monoclonal antibody were obtained from Sigma Chemical. *Clasto*-lactacystin β-lactone (*clasto*-LC), epoxomicin (Epox), and *p*-nitrophenylphosphate (PNPP) were from Calbiochem. Aminomethylcoumarin (AMC) and succinyl-Leu-Leu-Val-Tyr-methylcoumarylamide (Suc-LLVY-MCA) were from Peptide Institute. Anti-dinitrophenyl-KLH, rabbit IgG fraction, biotin-XX conjugate (anti-DNP-KLH antibody) was from Molecular Probes. Acetyl leucine chloromethyl ketone (ALCK) was synthesized from L-leucine chloromethyl ketone–HCl (LCK, Sigma Chemical) [16]. Mouse anti-vimentin (V9) monoclonal antibody was from Santa Cruz Biotechnology. Rabbit anti-Hsp70 polyclonal antibody was from Upstate Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (H + L) (FITC-anti mouse IgG antibody) and Texas red dye-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (Texas red-anti rabbit IgG antibody) were from Jackson Immuno Research Laboratories. COS-7-OPH cells that stably overexpressed C-terminal FLAG-tagged OPH were established as described previously [4]. Anti-OPH polyclonal antibody was obtained by immunizing rabbit with OPH purified from human red blood cells [10].

Cell culture. Cells were maintained in DMEM supplemented with 5% heat-inactivated FCS, penicillin (50 U/ml)/streptomycin (50 μg/ml), and G418 (1 mg/ml) and grown at 37 °C in a humidified 5% CO_2 atmosphere.

Assay for OPH activity. The OPH activity was measured as described previously [11]. Subconfluent layers of COS-7-OPH and the parental COS-7 cells were washed with PBS and lysed in PBS containing 0.5% NP-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 mM DTT, 20 mM β-glycerophosphate, and 10 mM PNPP. After normalization of protein content according to the protein assay, samples were immunoprecipitated with anti-OPH polyclonal antibodies. The immunoprecipitates were incubated with 0.5 mM AANA in 1.0 ml PBS at 37 °C for the indicated period. Absorbance of the supernatant of the reaction mixture at 405 nm was measured, and the concentration of released *p*-nitroaniline was determined using a molecular extinction coefficient of 7530 M^{-1} cm^{-1} [12].

Assay for proteasome activity. Proteasome activity was measured as described by Tsukahara [13]. Confluent layers of COS-7 cells were washed with PBS and lysed in 100 mM Tris–HCl (pH 8.0) containing 0.5% NP-40, 1 mM DTT. The resultant cell extracts (50 μg proteins) were incubated with substrate (100 μM Suc-LLVY-MCA) for 10 min at 37 °C. The fluorescence of AMC liberated by the hydrolysis of the substrate was measured at the excitation and emission wavelengths of 349 and 445 nm, respectively.

Quantification of protein carbonyls. Protein carbonyl contents were determined as described previously [4]. Briefly, COS-7-OPH and the parental COS-7 cells were incubated for 24 h after various treatments. The cells floating in the medium were collected by centrifugation. The remaining adherent cells were harvested and then combined with floating cells. The cells were lysed, and cell extracts were subjected to trichloroacetic acid (TCA) treatment. The resultant protein precipitates were dissolved in PBS and were derivatized with DNP. The DNP-derivatized proteins were subjected to enzyme-linked immunosorbent assay (ELISA) using anti-DNP-KLH antibody (1:1000 dilution in PBST) followed by the incubation with streptavidin–horseradish peroxidase. The immunocomplexes were visualized according to the manufacturer's instruction (TMB Microwell Peroxidase Substrate System) and the protein carbonyl levels were determined.

Determination of the densities of viable cells. COS-7-OPH and the parental COS-7 cells were seeded in each well of a 24-well plate at a density of 5×10^4 cells/well. After 24 h, cells were treated with paraquat in the presence or absence of proteasome inhibitors or ALCK. After further incubation for 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to quantify the density of viable cells [14].

Immunocytochemistry. COS-7-OPH cells were seeded on coverslips in 35-mm culture dishes at a density of 1.6×10^5 cells/dish and allowed to grow for 24 h. Then cells were treated with 5 μM Epox and further incubated for 24 h. The cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with methanol for 2 min, and blocked with 10% skim milk in PBS for 1 h. Cells on the coverslips were incubated with primary antibodies for 2 h. The coverslips were rinsed with PBS four times and then incubated for 1 h with FITC-labeled anti-mouse IgG antibody or Texas red-labeled anti-rabbit IgG antibody, as secondary antibodies. After extensive washings with PBS, 0.5 μg/ml Hoechst 33258 was added for nuclear staining. The coverslips were mounted and examined under a fluorescence microscope Leica model DM IRB (Leica Microsystems, Tokyo, Japan).

Results

In our previous study [4], we established COS-7-derived cells that stably overexpress OPH and termed them COS-7-OPH cells. COS-7-OPH cells showed significantly higher resistance to oxidative stress than COS-7 cells, suggesting that OPH has a crucial role in eliminating cytotoxic denatured proteins produced through oxidation. On the other hand, several lines of evidence indicate that the proteasome is responsible for protecting cells against oxidative stress by degrading oxidatively damaged proteins [5,6]. Therefore, we examined whether or not cellular resistance to oxidative stress is attenuated in COS-7-OPH cells treated with proteasome inhibitors.

Among several agents that inhibit proteosome activity, antibiotic lactacystin and its more potent derivative, *clasto*-LC, are believed to be highly specific proteasome inhibitors since they do not affect lysosomal proteases or calpains [15]. Significant inhibition of proteasome activity by *clasto*-LC was reproduced in our experiment (Fig. 1A). However, unexpectedly, *clasto*-LC strongly inhibited OPH activities (Fig. 1B). When cells were treated with *clasto*-LC, the levels of protein carbonyls were increased in control and paraquat-treated COS-7 and COS-7-OPH cells (Fig. 1C). Furthermore, in the presence of *clasto*-LC, COS-7-OPH cells were as sensitive as COS-7 cells to paraquat treatment (Fig. 1D). We examined various different agents known as proteasome inhibitors (data not shown), and found that Epox did not inhibit OPH while it almost completely blocked proteasome activity (Figs. 2A and B). In the presence of Epox, protein carbonyls were increased in both control and paraquat-treated COS-7 cells, indicating that the proteasome contributes to eliminate oxidized proteins (Fig. 2C). In the case of COS-7-OPH cells, Epox did not augment the level of paraquat-induced protein

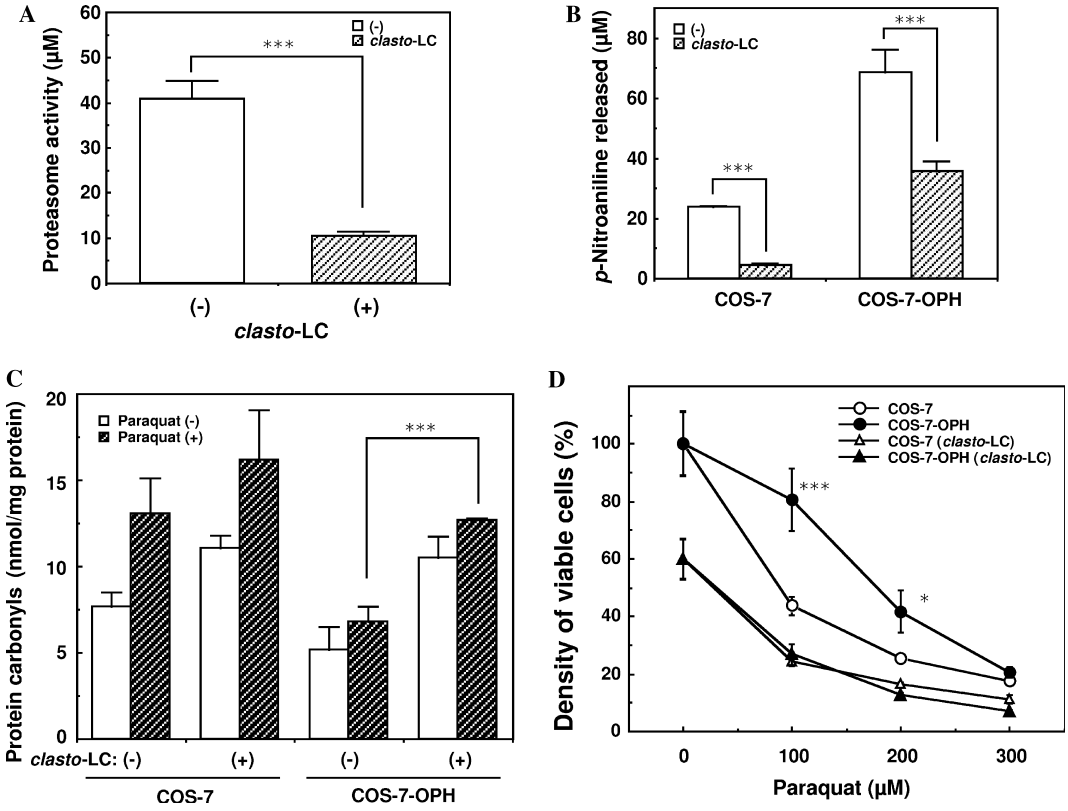

Fig. 1. *Clasto*-LC strongly impairs the cellular resistance to oxidative stress in COS-7-OPH cells by inhibiting both the proteasome and OPH. (A) COS-7 cells were treated with 10 μM *clasto*-LC for 24 h. Cell extracts were prepared and assay for proteasome activity was performed as described in Materials and methods. Significance of difference was determined by Student's *t* test (***$P < 0.005$). (B) COS-7 and COS-7-OPH cells were treated with 10 μM *clasto*-LC for 24 h. Cell extracts were prepared and assay for OPH activity was performed as described in Materials and methods (***$P < 0.005$). (C) COS-7 and COS-7-OPH cells were treated or left untreated with 10 μM *clasto*-LC for 24 h. Then cells were further incubated in the presence or absence of 250 μM paraquat for 24 h. The levels of protein carbonyls were determined as described in Materials and methods (***$P < 0.005$). (D) COS-7 and COS-7-OPH cells were treated or left untreated with 10 μM *clasto*-LC for 24 h. Then cells were treated with indicated concentrations of paraquat for 24 h. The densities of viable cells were determined as described in Materials and methods (*$P < 0.05$; ***$P < 0.005$ versus COS-7 cells, open circles).

carbonylation (Fig. 2C). Further, even in the presence of Epox, COS-7-OPH cells showed higher resistance to paraquat than COS-7 cells (Fig. 2D), suggesting that overexpressed OPH can compensate for the blockade of proteasomal degradation. As a strong inhibitor for acylpeptide hydrolase (APCH) activity of OPH, acetyl-leucine chloromethyl ketone (ALCK) has been characterized [16]. Indeed, ALCK did not significantly affect the proteasome activities in COS-7 and COS-7 OPH cells (Fig. 3A) while it strongly inhibited OPH (Fig. 3B). In the presence of ALCK, the level of protein carbonyls in paraquat-treated COS-7-OPH cells was significantly elevated, although it was still slightly lower than that in paraquat-treated COS-7 cells (Fig. 3C). Furthermore, the treatment with Epox plus ALCK resulted in the elevation of protein carbonyls in control and paraquat-treated COS-7-OPH cells as well as it did in COS-7 cells (Fig. 4A). COS-7 and COS-7-OPH cells showed similar sensitivity to paraquat under the condition where the proteasome and OPH were inhibited by Epox and ALCK, respectively, (Fig. 4B). These results suggest that both the proteasome and OPH are responsible for the elimination of oxidized proteins.

Misfolded protein aggregates resulting from genetic mutations, inappropriate protein assembly, and environmental stress are transported and removed from the cytoplasm by dynein motors via the microtubule network to a novel organelle termed aggresome where they are processed [9]. Inhibition of proteasome function prevents the degradation of misfolded proteins leading to their aggregation and deposition in aggresomes [17]. As shown in Fig. 5, OPH distributed diffusely throughout the cytoplasm in control COS-7-OPH cells, as revealed by the immunostaining using anti-FLAG or anti-OPH antibodies. In contrast, when cells were treated with Epox, OPH was strongly detected in a single large, juxtanuclear structure by both antibodies (indicated by white arrows in Fig. 5). The morphology and localization of Epox-induced OPH-enriched structure appear to resemble that of an aggresome. Therefore, we next compared the localization of OPH with those of known components of aggresomes [17,18]: the intermediate filament vimentin, γ-tubulin

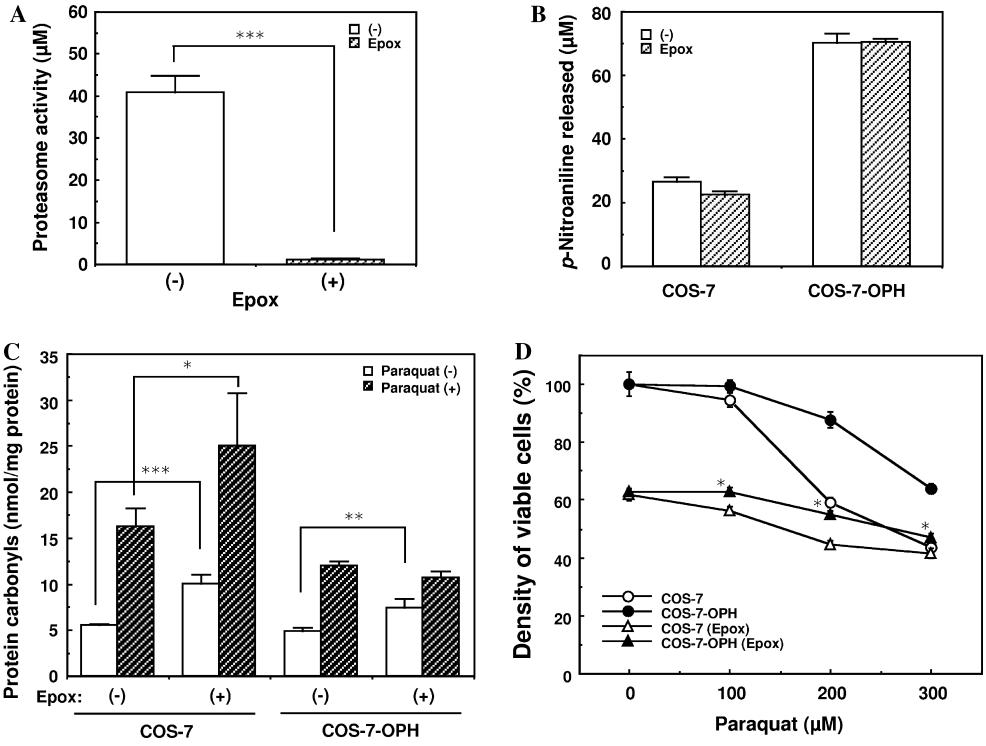

Fig. 2. COS-7-OPH cells retain cellular resistance to oxidative stress in a situation where the proteasome activity is selectively inhibited by the treatment with Epox. (A) COS-7 cells were treated with 1 μM Epox for 24 h. Cell extracts were prepared and assay for proteasome activity was performed as described in Materials and methods. Significance of difference was determined by Student's *t* test (***$P < 0.005$). (B) COS-7 and COS-7-OPH cells were treated with 5 μM Epox for 24 h. Cell extracts were prepared and assay for OPH activity was performed as described in Materials and methods. (C) COS-7 and COS-7-OPH cells were treated or left untreated with 1 μM Epox for 24 h. Then cells were further incubated in the presence or absence of 250 μM paraquat for 24 h. The levels of protein carbonyls were determined as described in Materials and methods (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.005$ versus control cells). (D) COS-7 and COS-7-OPH cells were treated or left untreated with 1 μM Epox for 24 h. Then cells were treated with indicated concentrations of paraquat for 24 h. The densities of viable cells were determined as described in Materials and methods (*$P < 0.05$ versus COS-7 cells treated with Epox, open triangles).

located at the microtubule-organizing center (MTOC), and Hsp70. As shown in the top panels (A–C) of Fig. 6, colocalization of OPH and vimentin was observed in Epox-treated COS-7-OPH cells using anti-OPH antibody. Similar colocalization was also observed between OPH and γ-tubulin (Figs. 6D–F). Using anti-FLAG antibody instead of anti-OPH antibody, aggresomal distribution of OPH was further confirmed by the merge staining with anti-Hsp70 antibody (Figs. 6G–I). Thus, OPH may contribute to degrade misfolded protein aggregates that exceed the capacity of the proteasome.

Discussion

Proteins within cells are continually being degraded to amino acids and replaced by newly synthesized proteins. Degradative machinery serves as a quality-control system that rapidly eliminates misfolded or damaged proteins whose accumulation would interfere with normal cell function and viability [5]. The most primary enzyme responsible for the degradation of misfolded proteins is the proteasome [5].

In eukaryotic cells, most proteins in the cytosol and nucleus are degraded via the ubiquitin–proteasome pathway [24]. The 26S proteasome contains a barrel-shaped proteolytic core complex (the 20S proteasome), capped at the ends by 19S regulatory complexes, which recognize ubiquitinated proteins. Recently, U-box proteins, which contain a domain (the U box) of about 70 amino acids, have been identified as a new type of ubiquitin ligase that serves as an enzyme for quality control of intracellular proteins [25]. Misfolded proteins are first recognized by molecular chaperons, and then either refolded by them or ubiquitinated by U-box proteins that interact with molecular chaperones [25]. The ubiquitinated misfolded proteins are degraded by 26S proteasome. In contrast, for the elimination of oxidatively denatured proteins, the 20S proteasome rather than the 26S proteasome may play an important role [6,19], although one still cannot rule out the possibility that the 26S proteasome takes part in the degradation of unfolded nonubiquitinated proteins produced through oxidation [7].

Besides the proteasome, we have characterized OPH as one of the critical enzymes responsible for the

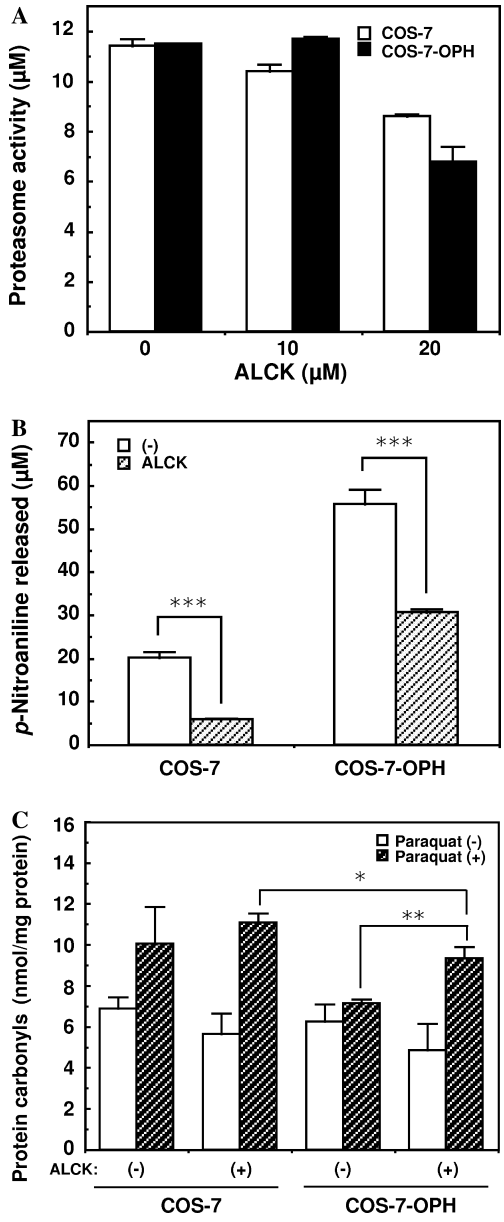

Fig. 3. ALCK inhibits OPH, resulting in the increased protein oxidation in COS-7-OPH cells. (A) COS-7 and COS-7-OPH cells were treated with indicated concentrations of ALCK for 24 h. Cell extracts were prepared and assay for proteasome activity was performed as described in Materials and methods. (B) COS-7 and COS-7-OPH cells were treated with 10 μM ALCK for 24 h. Cell extracts were prepared and assay for OPH activity was performed as described in Materials and methods. Significance of difference was determined by Student's *t* test (***$P < 0.005$). (C) COS-7 and COS-7-OPH cells were treated or left untreated with 10 μM ALCK for 24 h. Then cells were further incubated in the presence or absence of 250 μM paraquat for 24 h. The levels of protein carbonyls were determined as described in Materials and methods (*$P < 0.05$; **$P < 0.01$ versus control cells).

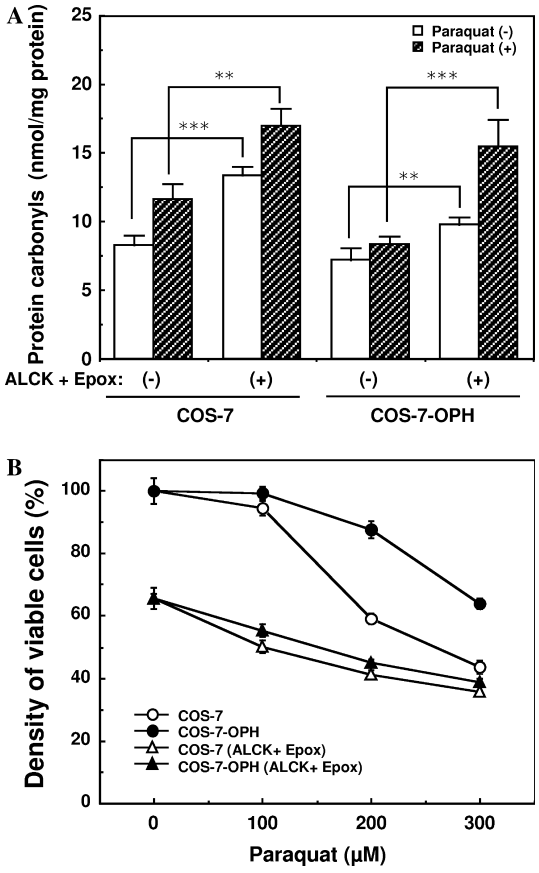

Fig. 4. Simultaneous treatment with Epox and ALCK abolishes the hyperactivity to eliminate oxidized proteins in COS-7 and COS-7-OPH cells. (A) COS-7 and COS-7-OPH cells were treated or left untreated with 1 μM Epox plus 10 μM ALCK for 24 h. Then cells were further incubated in the presence or absence of 250 μM paraquat for 24 h. The levels of protein carbonyls were determined as described in Materials and methods. Significance of difference was determined by Student's *t* test (**$P < 0.01$; ***$P < 0.005$ versus control cells). (B) COS-7 and COS-7-OPH cells were treated or left untreated with 1 μM Epox plus 10 μM ALCK for 24 h. Then cells were treated with indicated concentrations of paraquat for 24 h. The densities of viable cells were determined as described in Materials and methods.

elimination of oxidatively damaged proteins [4]. Interestingly, inhibition of proteasome activity by Epox caused a significant elevation of paraquat-induced protein carbonylation in parental COS-7 cells whereas it did not in COS-7-OPH cells. Epox is known to modify four catalytic subunits of the 20S proteasome [20], therefore, our results indicate that overexpressed OPH can substitute for the 20S proteasome to eliminate oxidatively damaged proteins. Reinheckel et al. [19] showed that the 20S proteasome is more resistant to oxidative stress than the ATP- and ubiquitin-dependent 26S proteasome. OPH also showed higher resistance to oxidative stress as the 20S proteasome did (unpublished result). Strongly oxidized proteins are known to be poor substrates for the proteasome, since they easily form covalent cross-links and aggregates [21]. OPH may play a role in the clearance of such oxidized proteins that are poor substrates for the proteasome.

As aggregated proteins are toxic, failure to degrade misfolded and aggregated proteins is a dominant contributing factor to neuronal cell death in many neurodegenerative diseases [22]. Aggresomes were first

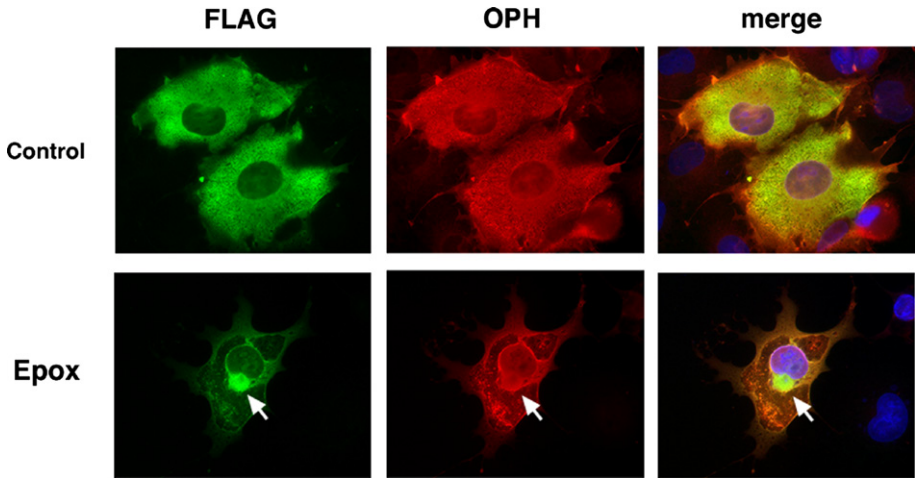

Fig. 5. Inhibition of the proteasome activity results in a single prominent juxtanuclear translocation of OPH. COS-7-OPH cells were treated or left untreated with 5 μM Epox for 24 h. Then cells were immunostained with anti-FLAG (green) or anti-OPH (red) antibodies as described in Materials and methods. The nuclei were counterstained with Hoechst33258 (blue).

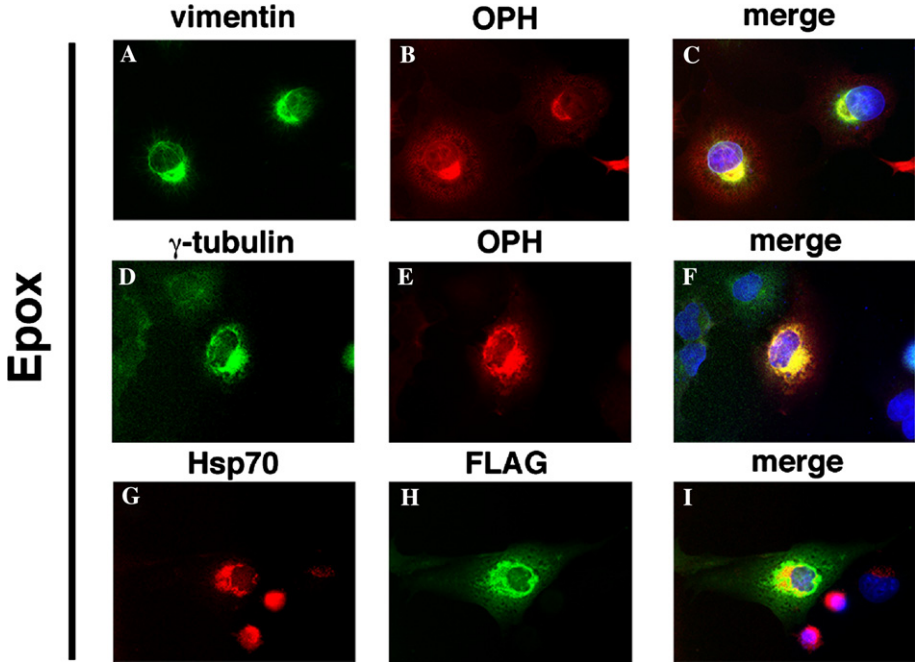

Fig. 6. Colocalization of OPH with vimentin, γ-tubulin, and HSP70 in aggresomes. COS-7-OPH cells were incubated with 5 μM Epox for 24 h. OPH was immunostained with anti-OPH (B and E, red) or with anti-FLAG (H, green). As makers for aggresomes, vimentin (A, green), γ-tubulin (D, green), and Hsp70 (G, red) were double-immunostained with OPH. The nuclei were counterstained with Hoechst33258 (blue). Overlaid images (merge) demonstrate the colonization of OPH with various aggresome markers (C, F, and I).

identified in the characterization of a mutant form of the cystic fibrosis transmembrane conducting regulator (CFTR), which is prone to misfolding and aggregation [9]. A role for aggresomes in accelerating the turnover of misfolded protein is suggested by their composition. In addition to sequestering misfolded proteins, aggresomes extensively recruit cytosolic pools of proteolytic machinery, such as ubiquitin and major components of the 26S proteasome including the 20S proteasomal subunits and the PA700 and PA28 proteasomal activator complexes [26]. Aggresomes are also rich in molecular chaperones including members of Hsp40 family, a member of the chaperonin family (TCP1), and Hsp70 [26,27]. These observations suggest that aggresomes may represent a site where highly concentrated proteasome activity exists, leading to the effective protection of cells against excess amounts of misfolded proteins. Alternatively, aggresomes may provide access to other proteolytic pathways in situations where the capacity of the proteasome is exceeded. Indeed,

aggresome formation is observed when cells are treated with proteasome inhibitors [17].

In COS-7-OPH cells, Epox treatment did not interfere with OPH activity (Fig. 2B), while it significantly induced the aggresomal translocation of OPH (Fig. 6). Although direct evidence has not been provided yet, the catalytically active OPH may be involved in the clearance of misfolded proteins accumulated in aggresomes that are formed in proteasome-inhibited cells. Since OPH showed chymotrypsin-like endoprotease activity toward oxidized proteins leaving relatively larger sizes of peptide fragments in vitro [23], other types of peptidases besides OPH and the proteasome may also participate in the complete degradation of misfolded proteins into amino acids. Thus, coordination of multiple proteolytic enzymes, such as the proteasome and OPH, can facilitate elimination of harmful proteins and may be relevant to a wide array of diseases caused by misfolded protein accumulation.

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