Enhancement of Ubiquitin Conjugation Activity Reduces Intracellular Aggregation of V76D Mutant γD-Crystallin

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PURPOSE. The ubiquitin–proteasome pathway (UPP) is an important protein quality control mechanism for selective degradation of abnormal proteins. The objective of this study was to test the hypothesis that enhancement of the UPP capacity could attenuate the accumulation and aggregation of misfolded proteins using V76D-γD-crystallin as a model substrate.

METHODS. Wild type (wt) and V76D mutant γD-crystallin were fused to red fluorescence protein (RFP) and expressed in human lens epithelial cells. The cellular distribution of the expressed proteins was compared by fluorescence microscopy. The solubility of wt- and V76D-γD-crystallin was determined by cellular fractionation and Western blotting. Wt-γD-RFP and V76D-γD-RFP were also cotransfected along with a ubiquitin ligase (CHIP) or a ubiquitin-conjugating enzyme (Ubc5) into cells. Levels of wt- and V76D-γD-crystallin, the percentage of transfected cells with aggregates, and aggregate size were quantified and compared among different groups.

RESULTS. Wt-γD-crystallin was evenly distributed in cells, whereas V76D-γD-crystallin formed intracellular aggregates. Eighty percent of wt-γD-crystallin was detected in the soluble fraction, whereas only 7% of V76D-γD-crystallin was soluble. CHIP or Ubc5 coexpression reduced the protein level of V76D-γD and concomitantly its aggregation in transfected cells; these effects could be attenuated by proteasome inhibitor. Mutant CHIP with defect TPR (tetrapeptide repeat) or U-box domain failed to reduce levels of V76D-γD-crystallin.

CONCLUSIONS. Enhancing ubiquitin conjugation activity reduces accumulation and aggregation of V76D-γD-crystallin by promoting its degradation. Upregulation of ubiquitin-conjugating activity could be an effective strategy to maintain lens transparency by eliminating other forms of misfolded proteins.


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PROTEIN aggregation is a prevalent phenomenon observed in various diseases. Several human disorders are related to intracellular or extracellular protein aggregates. Among these are cataracts as well as neurodegenerative diseases such as Parkinson’s disease, Huntington’s disease, and Alzheimer’s disease.1-4 Although the proteins that are causally related to these diseases have unrelated functions, a common feature shared by these diseases involves the transformation of native functional proteins into aggregates.

Lens cells contain high concentrations of proteins, the majority of which are structural proteins called crystallins. Among them are eight γ-crystallin genes,5 of which the most abundant are γC- and γD-crystallins.6-8 Point mutations in these two lens crystallins are the common genetic basis for many reported human autosomal dominant cataracts, many of which result in reduced solubility and protein aggregation in lens cells.9-15 Point mutations in γ-crystallin-encoding genes have also been identified as causes of cataract in mice. For example, missense mutation in the γD-crystallin gene, resulting in the substitution of a valine (V) at codon 76 by an aspartic acid (V76D) has been found to cause cataracts.16 The V76D-γD mutant reduces protein solubility in lens and forms substantial intranuclear aggregates that cause lens opacification and disrupt the denucleation process of inner lens fiber cells.17

The ubiquitin–proteasome pathway (UPP) is a primary protein quality control system that selectively degrades aberrant proteins to maintain cellular protein homeostasis. Protein degradation via the UPP is a multistep process. The reaction begins with activation of ubiquitin by the ubiquitin-activating enzyme (E1). Subsequently, a ubiquitin conjugate enzyme (E2) acquires the activated ubiquitin from E1. A ubiquitin-protein ligase (E3) recruits a protein destined for degradation and guides the transfer of the activated ubiquitin from the E2 to the substrate.18 Once a protein is tagged with one ubiquitin molecule, additional ubiquitin molecules may be attached to the previous ubiquitin and form polyubiquitin chains. Finally, the polyubiquitinated protein is recognized and degraded by the proteasome.19

Numerous studies indicate that impairment of the UPP by inhibition of the proteasome, or knockdown/knockout of protein quality control E3s including the C-terminus of Hsp70 interacting protein (CHIP), promotes the accumulation and aggregation of mutant or other forms of damaged proteins.20-25 These studies clearly indicate that a functional UPP is essential for timely clearance of abnormal proteins and preventing their accumulation and aggregation. However, only a few studies have attempted to enhance UPP activity to promote the clearance of such damaged proteins, because it is much more difficult to enhance the UPP capacity than to inhibit UPP activity. One report showed that overexpression of a specific proteasome subunit, S5a, could promote clearance of oxidized proteins,26 but the effects were marginal. A recent study showed that inhibiting a proteasome-associated deubiquitinating enzyme, USP14, could promote the degradation of many...
typical substrates of the UPP and enhance the clearance of oxidized proteins.27

In most types of cells or tissue, including lens epithelial cells and fibers, ubiquitination is the substrate-selecting and limiting step for UPP-mediated degradation. Enhancing the ubiquitinating activity would be more effective than manipulating the proteasome in promoting the degradation of abnormal proteins. Consistently, increasing the expression of CHIP promoted the ubiquitination and degradation of wild type (wt) and polyglutamine-expanded ataxin-1, offering protection from their neurotoxicity.28 However, such studies in mammalian cells or tissues are scarce, and further investigation is warranted, particularly in lens cells where accumulation of damaged proteins is causally associated with cataract formation.

Ubc5 is a family of closely related ubiquitin-conjugating enzymes that include UbcH5a, UbcH5b, and UbcH5c. All these Ubc5 isozymes have been shown to support the degradation of abnormal proteins.29 In the present study, only UbcH5a was used. CHIP is known to be one of the cognate E3s for Ubc5, which is a ubiquitin ligase that bridges chaperone function with the degradation of damaged or unstable proteins.30–32 CHIP binds Hsp70 or Hsp90 through its N-terminal TPR (tetra-tricopeptide repeat) domain, and binds E2 via its C-terminal U-box domain.33 By recruiting a specific E2, such as Ubc5, CHIP mediates ubiquitination and selective degradation of many labile proteins chaperoned by Hsp70 or Hsp90. These substrates include proto-oncogene products, kinases, and nuclear hormone receptors.34 Notably, some aggregation-prone proteins, such as α-synuclein and tau, both of which are involved in neurodegenerative diseases, are also clients of CHIP.35

We have demonstrated in previous studies that lens cells, including lens fibers in the lens nuclear region, have a functional UPP.37–42 However, the capacity of the UPP in lens fibers decreases with aging. Supplementation of Ubc5 to the supernatants of lens epithelial cells and lens fibers, including the fibers in the lens nuclear region, can partially restore the ubiquitin-conjugating activity and has been shown to increase the degradation of damaged or modified proteins.42,43 In this study, we determined whether enhancing the ubiquitin-conjugating activity in living lens cells could reduce the accumulation and aggregation of misfolded or damaged proteins. V76D mutant γD-crystallin was used as a model substrate to address this issue.

Consistent with the data from V76D mutant mice,17 we found this mutant γD-crystallin mainly in the insoluble fraction and found that it formed perinuclear or intranuclear aggregates when expressed in human lens epithelial cells (HLEC) and HeLa cells. When the ubiquitination conjugation activity was enhanced by overexpressing CHIP or Ubc5, the ratio of transfected cells with V76D-γD aggregates, as well as the size of aggregates, was reduced. In addition, overexpression of CHIP and Ubc5 significantly reduced the levels of V76D-γD-crystallin in the insoluble fraction. Conversely, inhibition of the UPP by the proteasome inhibitor MG132 increased the number and size of the aggregates of V76D-γD-crystallin, as well as the level of this mutant protein in cells. In comparison, overexpression of Ubc5 is more effective than overexpression of CHIP in promoting the degradation of V76D-γD-crystallin and suppressing its intracellular aggregation.

**Materials and Methods**

**Plasmid Construction**

CHIP and Ubc5 cDNAs were originally constructed using pcDNA3.1 as vector. UbcH5a and His-tagged CHIP cDNA fragments were released from the original vector by restriction digestion using NorI and EcoRI. The NorI overhang was filled in by T4 DNA polymerase. Digestion products were analyzed on a 1% agarose gel (for His-tagged CHIP) or 1.5% agarose gel (for UbcH5a) and purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Purified DNA fragments that encode His-tagged CHIP or UbcH5a were inserted into the EcoRI and BamHI sites of the pIRE52-EGFP vector. These constructs express CHIP or Ubc5, along with EGFP, which allows us to track the CHIP or Ubc5 expression in cells. The recombinant clones were confirmed by restriction enzyme digestion and direct sequencing. Complementary DNAs of wt- and V76D-γD-crystallin were subcloned into the EcoRI and BamHI sites of the pDsRedN1 vector, with the 3′ ends of cDNAs in-frame with the red fluorescent protein (RFP) gene as previously described.44 The cDNAs of two types of CHIP mutants, c-myc-K30A (ΔTPR) and c-myc-H262Q (ΔU-box), were subcloned into the pcDNA3.1 vector as described previously.35

**Cell Culture and Cotransfection Experiments**

HLEC, the human lens epithelial cell line SRAS01/04, and HeLa cells were cultured at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂. For Western blotting, the cells (8 × 10⁴) were seeded in 60 mm culture plates one day before transfection. For fluorescence microscopic imaging, the cells (8 × 10⁴/chamber) were seeded in four-chamber culture slides. Transfection was carried out using PolyFect (Qiagen) and/or ER-EGFP (Invitrogen, Grand Island, NY) following manufacturer’s protocol. To compare the solubility and cellular distribution, HLECs and HeLa cells were transfected with plasmids that express wt-γD-crystallin-RFP or V76D-γD-crystallin-RFP. To determine the effects of CHIP or Ubc5 on degradation of wt- and V76D-γD-crystallins, the cells were cotransfected with plasmid that expresses γD-crystallin-RFP and plasmids that express CHIP/EGFP or Ubc5/EGFP at 1:1 ratio. Empty pIRE52-EGFP (the plasmid that expresses only EGFP) was used as the control for CHIP and Ubc5 in the cotransfection experiments. To study the effects of proteasome inhibitor, 28 hours after transfection, the cells were treated with 10 μM MG132 for 16 hours. To determine whether Hsp70/90 chaperones are involved in identifying V76D as a substrate, the cells were cotransfected with plasmid that expresses V76D-γD-crystallin-RFP and plasmids that express wt CHIP TPR-mutant CHIP, or U-box-mutant CHIP at 1:1 ratio. Empty pcDNA3.1 vector was used as the control in the cotransfection experiments.

**Fluorescence Microscopy and Quantification of Aggregates**

The transfected cells in four-chamber culture slides were washed with PBS and fixed using 4% paraformaldehyde for 15 minutes at room temperature. The cells were washed with PBS three times and stained with 4′,6-diamidino-2-phenylindole (DAPI) for 2 minutes. After three washes with PBS to remove excess dye, the slides were mounted using Mounting Medium (Dako). The transfected cells in four-chamber culture slides were viewed using a microscope (Zeiss, Oberkochen, Germany).

To compare the fluorescence intensity in different groups, the peak intensity, as well as the accumulated intensity of RFP or GFP fluorescence per individual cell, was measured, respectively. One hundred cells were selected randomly from each group. For peak intensity, the ratio (RFP/GFP) in each cell was evaluated, and the rank distribution of the ratio in each group was graphed.

To quantify the number of transfected cells with aggregates, five 10X views, each containing approximately 300 cells, were selected randomly from each group, and the photographs were analyzed in a double-blinded manner.

**Preparation of Insoluble and Soluble Fractions**

Forty-four hours after transfection, the cells were washed and scraped in cold PBS. The cells were then centrifuged at 3000 rpm for 3 minutes.
The supernatant was discarded, and the pellets were lysed in 1% NP-40, 10 mM pH 7.5 Tris-HCl, 100 mM KCl, 1 mM EDTA, 5 mM MgCl2, 2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 10 mM N-ethylmaleimide. Samples were centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant, which contained the soluble proteins, was transferred to another Eppendorf tube, and equal volume of 2X SDS-gel loading buffer was added into each soluble sample. For the pellet containing insoluble proteins, two volumes of 1X SDS-gel loading buffer were added into each sample, so that the soluble fraction and insoluble fraction contained the same final volume for each sample. All the samples were boiled for 5 minutes and analyzed by SDS-PAGE and subsequent immunoblotting.

**SDS-PAGE and Western Blotting**

The samples dissolved in SDS loading buffer were separated on SDS-PAGE and blotted onto nitrocellulose membrane. Nonspecific binding sites were blocked with blocking solution (0.05% Tween 20 and 2.5% nonfat milk in Tris-buffered saline [TBS]; Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature. The membrane was incubated with primary antibody in blocking buffer overnight at 4°C, then washed five times (5 minutes for each wash) with TBST (0.05% Tween 20 in TBS) to remove the unbound antibody. The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour at room temperature. The excess secondary antibody was removed by washing the membrane five times (5 minutes each) with TBST, and the bands were visualized using the Super Signal Kit (Thermo Scientific, Rockford, IL). RFP-wt-γ-D-crystallins and RFP-V76D-γ-D-crystallins were detected using anti-RFP (1:1000; Living Colors DsRed Polyclonal Antibody; Clontech, Mountain View, CA). (His)6-tagged CHIP was detected with mouse monoclonal anti-His antibody (1:500; GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Ubc5 was detected with rabbit polyclonal antibody (1:1000; Boston Biochem, Cambridge, MA). C-myc-K30A and c-myc-H262Q were detected with mouse monoclonal anti-myc tag (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). To confirm equal protein loadings for insoluble fractions, the levels of the nuclear envelope protein lamin A/C were detected using the mouse monoclonal antibody (1:200, Novacastro; Leica, Buffalo Grove, IL). The levels of β-actin were monitored with mouse monoclonal antibody (1:1000; Sigma-Aldrich) as loading control for the soluble fractions. HRP-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) were used as secondary antibodies.

The developed films were scanned, and the images were quantified with ImageJ 1.45 (provided in the public domain by the National Institutes of Health, http://rsweb.nih.gov/ij/download.html).

**Statistical Analysis**

Data were expressed as the mean and standard deviation, representing an average of three independent experiments. One-way analysis of variance (ANOVA) was used to compare means from different groups. P < 0.05 was considered to be statistically significant.

Data on RFP/GFP ratio were described with median and rank distribution, since these data do not meet the criteria of normalized distribution.

**Results**

**V76D-γ-D-Crystallin Forms Nuclear or Perinuclear Aggregates in HLEC and HeLa Cells**

To compare the intracellular distribution of wt- and V76D-γ-D-crystallins, HLEC were transfected with either wt-γ-D-crystallin-RFP or V76D-γ-D-crystallin-RFP, and the distribution of the fusion proteins was observed with fluorescence microscopy. The wt-γ-D-crystallin was found to be uniformly distributed throughout the cell (Fig. 1A, upper panels), indicating that fusing RFP to the crystallin did not perturb the usual solubility of γ-D-crystallin. By contrast, the V76D mutant γ-D-crystallin formed aggregates of different sizes, mainly in the nuclear or perinuclear regions of the transfected cells (Fig. 1B, upper panels).

To determine whether the wt and mutant γ-D-crystallin in other types of cells behave similarly to the way they do in HLEC, we transfected HeLa cells with the same plasmids. Compared to HLEC, HeLa cells offer higher transfection efficiency and higher expression levels, which are essential for mechanistic studies. The cellular distributions of wt- and V76D-γ-D-crystallins in HeLa cells (Figs. 1A, 1B, lower panels) were comparable to those observed in HLEC. Whereas wt-γ-D-crystallin-RFP fusion was evenly distributed in the cell body, the majority of the V76D-γ-D-crystallin-RFP fusion was found in nuclear or perinuclear aggregates.

We further analyzed the localization of V76D-γ-D-crystallins by confocal imaging in both HLEC and HeLa. Confocal microscopy (Fig. 2 and Supplementary Fig. 1, http://www.iovs.orglookup/suppl/doi:10.1167/iovs.12-9744/-/DSupplemental) shows that the majority of the perinuclear aggregates were colocalized with the endoplasmic reticulum (ER), while a relatively small amount of mutant proteins was localized in the nucleus. It is not clear why this mutant cytoplasmic protein was colocalized with ER. This mutant protein might be tangled with ER-membrane proteins and bound onto the ER membrane. This method cannot distinguish whether the mutant protein is localized on ER surface or in ER lumen.

The above data show that V76D-γ-D-crystallin is prone to form nuclear/perinuclear aggregates in both lens and nonlens cells. This provides an opportunity to biochemically investigate how the UPP affects the aggregation of V76D-γ-D-crystallin using a cell line with which high efficiency of transfection can be achieved.

**V76D Mutation Reduces the Solubility of γ-D-Crystallin in Cells**

To study the change in solubility due to V76D mutation, the transfected HeLa cells were harvested and the proteins separated into insoluble and soluble fractions. Levels of wt- and V76D-γ-D-crystallins in the soluble and insoluble fractions were determined by Western blotting. As shown in Figure 3, ~80% of wt-γ-D-crystallin was found in the soluble fraction; however, only ~7% of the V76D-γ-D-crystallin was found in the soluble fraction. These data indicate that the V76D mutation dramatically reduces the solubility of γ-D-crystallin and that insolubility may contribute to the aggregation of the V76D mutants in cells.

**CHIP or Ubc5 Overexpression Reduces Intracellular Aggregates of V76D-γ-D-Crystallin**

CHIP is an important protein quality control E3, and Ubc5 is the cognate E2 for the reaction. Both CHIP and Ubc5 have been reported to be involved in the elimination of aggregation-prone proteins.46 Previously, we found that levels of CHIP and Ubc5 were limited in cells, and supplementation of CHIP or Ubc5 enhanced the degradation of denatured proteins and other forms of modified proteins.47 To test whether overexpression of CHIP or Ubc5 could have beneficial effects in reducing V76D-γ-D-crystallin aggregates, cells were cotransfected with V76D-γ-D-crystallin together with either CHIP or Ubc5. As shown in Figure 4, overexpression of CHIP or Ubc5 significantly reduced the percentage of transfected cells with V76D-γ-D-crystallin aggregates. In HLEC, ~56% of transfected cells in the control group formed intracellular aggregates (Fig.
4A, left panel). When CHIP or Ubc5 was coexpressed in the cells, the percentages of transfected cells with aggregates were reduced to ~34% and ~26%, respectively. In HeLa cells, 37% of V76D-γD-crystallin–transfected cells contained aggregates in the control group, whereas only 23% and 13% contained aggregates in the CHIP and the Ubc5 cotransfection groups, respectively (Fig. 4B, left side). These data indicate that enhancing the capacity of UPP by overexpression of CHIP and Ubc5 can reduce V76D-γD-crystallin aggregation. It appeared that overexpression of Ubc5 was more effective than overexpression of CHIP in reducing the V76D-γD-crystallin aggregation. Consistent with the role of UPP in suppressing the aggregation of V76D mutant γD-crystallin, treatment with proteasome inhibitor (MG132) partially reversed the effects of CHIP and Ubc5, both in HLEC and in HeLa cells (Figs. 4A, 4B, right panels).

Aggregate size is closely related to the light-scattering property of damaged/mutant protein. Smaller aggregates have less effect on lens transparency, though they may cause light scattering at shorter wavelengths. Therefore, it is of importance to study whether CHIP and Ubc5 can reduce the aggregate sizes of the mutant crystallin. Figure 5 shows representative images of cells with V76D-γD-crystallin aggregates in different groups. The data clearly showed that overexpression of CHIP or Ubc5 not only reduced the proportion of cells with aggregates, but also reduced the size of intracellular aggregates in both HLEC (Fig. 5A) and HeLa cells (see Supplementary Fig. 3A, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental). Attesting to the fact that the diminution of aggregates is due to enhanced degradation, when cells were treated with proteasome inhibitor, there was a dramatic increase in the percentage of V76D-γD-crystallin aggregates as well as in the size of intracellular aggregates (Fig. 5B and Supplementary Fig. 3B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental). We then measured the peak intensity of RFP and GFP fluorescence in each individual cell and determined the RFP/GFP ratio. Since protein aggregation results in high fluorescence intensity, the peak intensity of RFP in cells is informative for protein aggregation. Diminution
of aggregates when CHIP or Ubc5 was overexpressed was further illustrated by relatively lower RFP/GFP in these groups (Fig. 5C and Supplementary Fig. 3C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental).

For lens cells, the medians of the RFP/GFP ratio for the control, the CHIP, and the Ubc5 groups were 0.62, 0.45, and 0.42, respectively. After treatment with MG132, the medians of the ratio for the control, the CHIP, and the Ubc5 were 0.86, 0.95, and 0.90, respectively. We have observed similar changes for RFP/GFP ratios in HeLa cells (see Supplementary Fig. 4, panel C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental), indicating that the level of fluorescent proteins in individual cells is mainly determined by the ability of the cell to take up plasmids.

Overexpression of CHIP or Ubc5 Decreases Levels of V76D-γD-Crystallin in the Insoluble Fraction of the Cells

In order to confirm that overexpression of CHIP and Ubc5 promotes the degradation of V76D-γD-crystallin, we determined the effects of overexpression of CHIP or Ubc5 on levels of V76D-γD-crystallin in the soluble and insoluble fractions of transfected HeLa cells. Data in Figure 6C showed that overexpression of CHIP or Ubc5 reduced levels of V76D mutant γD-crystallin in the insoluble fractions. Densitometric analysis revealed that, as compared to the control, overexpression of CHIP and Ubc5 decreased the levels of V76D-γD-crystallin in the insoluble fraction by 30% and 60%, respectively (Fig. 6D).

The effects of expression of CHIP or Ubc5 on levels of V76D-γD-crystallin in the soluble fractions were less obvious (Fig. 6A). Although overexpression of CHIP seemed to increase the proportion of V76D-γD-crystallin in the soluble fractions, the increase was not statistically significant (Figs. 6A, 6B). The marginal increase in levels of V76D-γD-crystallin in the soluble fraction may be related to increased refolding capacity, as overexpression of CHIP triggers heat shock responses.49

Consistent with the degradation of mutant proteins by the UPP, inhibiting the proteasome with MG132 resulted in accumulation of V76D-γD-crystallin in both soluble and insoluble fractions (Fig. 6). Treatment of the cells with MG132 also diminished the effects of CHIP or Ubc5 on levels of V76D-γD-crystallin in the insoluble fractions (Fig. 6D).

Concurrent expression of CHIP and Ubc5 also significantly reduced the aggregation of V76D mutant γD-crystallin to a similar extent as overexpression of CHIP or Ubc5 alone (see Supplementary Figs. 4A, 4B, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental). The rapid degradation of the mutant protein upon simultaneous overexpression of CHIP and Ubc5 resulted in lower levels of mutant proteins in transfected cells (see Supplementary Fig. 4C, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental); some of them may be below the detecting limit. Thus, the percentage of transfected cells with...
aggregates was probably overestimated due to reduced number of cells with detectable mutant γD-crystallins.

**Overexpression of CHIP and Ubc5 Has Little Effect on Levels of Wild Type γD-Crystallins in Cells**

To determine whether the effect of overexpressed CHIP or Ubc5 on levels of V76D-γD-crystallin was specific to mutant proteins, the levels of wt-γD-crystallins in the soluble and the insoluble cellular fractions were determined when coexpressed with CHIP or Ubc5. Consistent with the data in Figure 3, the vast majority of the wt-γD-crystallin was found in the soluble fraction (Fig. 7A). Overexpression of CHIP had little effect on levels of wt-γD-crystallin in the soluble fraction (Figs. 7A, 7B). However, overexpression of Ubc5 resulted in a small, but reproducible, decrease in levels of wt-γD-crystallin in the soluble fraction. Due to the low levels of wt-γD-crystallin in the insoluble fraction, variations from experiment to experiment were relative high as compared to the variations in the soluble fractions. Overexpression of Ubc5, but not CHIP, modestly decreased the levels of wt-γD-crystallin in the insoluble fraction (Figs. 7C, 7D), but the difference was not statistically significant. Inhibition of the proteasome by MG132 had no detectable effects on levels of wt-γD-crystallin in the soluble fraction, but resulted in a significant increase in levels of wt-γD-crystallin in the insoluble fraction (Figs. 7C, 7D).

**The Chaperone System Is Involved in the Recognition and Degradation of V76D Mutant γD-Crystallin**

CHIP binds chaperones Hsp70 or Hsp90 through its N-terminal TPR domain, and binds E2 via its C-terminal U-box domain. To determine whether Hsp70/90 are involved in CHIP-
mediated degradation of V76D-γD-crystallin, we compared the effects of wt CHIP and TPR domain mutant CHIP (ΔTPR), which abolished the ability to interact with Hsp70 and Hsp90, or U-box mutant CHIP (ΔU-box), which abolished the ability to catalyze ubiquitination, in reducing V76D-γD-crystallin aggregation.

Both ΔTPR and ΔU-box failed to reduce V76D aggregation (Fig. 8A). Western blot showed that increasing CHIP reduced the protein level of V76D-γD-crystallin especially in the insoluble fraction, whereas the levels of V76D-γD-crystallin in the ΔTPR and the ΔU-box groups were comparable to that in the control group (Figs. 8B, 8C). This result suggests that the Hsp70 or Hsp90 is involved in the CHIP-mediated degradation of this mutant crystallin.

**DISCUSSION**

Disruption of the proper arrangement of lens crystallins is causally related to cataractogenesis. Mutations of lens proteins, including crystallins, are the major causes of congenital cataracts. Accumulation of damaged or mutant proteins in lens cells not only causes light scattering, but also interferes with other cellular functions by tangling or sequestering other proteins. Previous studies have demonstrated that the UPP plays an important role in selective degradation of various forms of damaged or nonnative proteins. Rapid degradation of damaged or mutant proteins could prevent their accumulation and aggregation.

In this work we determined the effects of enhanced UPP activity on accumulation and aggregation of a cataract-causing mutant crystallin, V76D-γD-crystallin. Consistent with previous work, we found that the V76D-γD-crystallin formed perinuclear/intranuclear aggregates in both lens cells and nonlens cells, indicating that the aggregation was due to the properties of the mutant protein, regardless of the types of cells in which it is expressed. Enhancing the UPP capacity by overexpression of CHIP or Ubc5 suppressed the aggregation of V76D-γD-crystallin by reducing its levels in cells. Furthermore, inhibition of the proteasome resulted in accumulation of wt-γD-crystallin in the insoluble fraction, indicating that a small fraction of wt-γD-crystallin, probably in a denatured or modified form, was also degraded by the UPP. These data clearly indicate that a functional UPP is required for clearance of aggregation-prone abnormal proteins, and that enhancing the UPP capacity in cells can promote the degradation of abnormal proteins and prevent their accumulation and aggregation.

The selective degradation of abnormal proteins has been known for a long time, but the mechanism by which the UPP distinguishes abnormal from native proteins remains an unsolved mystery. It is believed that the E2s and E3s are jointly responsible for substrate recognition and ubiquitination. Moreover, recent studies indicate that molecular chaperones, in particular Hsp90/Hsp70, are utilized by the UPP to recognize abnormal proteins. CHIP is one of the E3s that selectively target chaperone-bound substrates for degra-
FIGURE 7. Overexpression of CHIP and Ubc5 did not significantly reduce the amount of wild type γD-crystallin. HeLa cells were cotransfected with wt-γD-crystallin and either vector alone, CHIP, or Ubc5. The soluble (A) and insoluble (C) fractions were separated and resolved by SDS-PAGE and probed using anti-RFP. The bands identified by the antibody were then quantified with ImageJ (B, D). Overexpression of CHIP did not affect the amount of wt-γD-crystallin; overexpression of Ubc5 reduced the level of wt-γD-crystallin, but not in a statistically significant manner.

FIGURE 8. The effects of CHIP require functional TPR and the U-box domains. Cells were transfected with RFP-V76D-γD-crystallin and vector alone, wild type His-tagged-CHIP, c-myc-ΔTPR-CHIP, or c-myc-ΔU-box-CHIP, respectively. (A) Nuclei were stained with DAPI, and the aggregation of V76D in each group was compared with fluorescence microscopy. The soluble (B) and insoluble (C) fractions were separated by SDS-PAGE and probed using anti-RFP antibody to compare the level of V76D-γD-crystallin.
Supplementation of CHIP or Ubc5 to lens Hsp90 was involved in identifying V76D-crystallin as a model substrate, suggesting that Hsp70 or Hsp90 was involved in identifying V76D-crystallin for degradation.

Our previous work indicates that levels of CHIP and Ubc5 in lens cells are limited. Supplementation of CHIP or Ubc5 to lens supernatants, including supernatants of lens fibers, enhances the degradation of damaged proteins.13 This work extends prior observations by demonstrating that increasing cellular levels of CHIP or Ubc5 can enhance protein ubiquitination (see Supplementary Fig. 5, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9744/-/DCSupplemental) and accelerate the degradation of mutant crystallins, preventing their aggregation. However, the balance between refolding and the degradation of damaged proteins is important for proteome homeostasis. Overexpression of CHIP or Ubc5 could tip the balance and result in degradation of some repairable proteins. Extensive degradation may even cause function loss of some unstable proteins. For example, premature degradation of inefficiently folded mutant cystic fibrosis transmembrane conductance regulator (CFTR) (ΔF508) causes cystic fibrosis.62 We also observed that overexpression of Ubc5 not only promoted the degradation of mutant crystallin, but also reduced the levels of wt crystallin. To gain the maximal benefits from manipulation of the UPP, levels of CHIP, Ubc5, and other rate-limiting components of the UPP must be fine-tuned. Future studies on the dose-dependent effects of CHIP and Ubc5 on degradation of wt and mutant crystallins are necessary to determine the optimal levels of CHIP and Ubc5 in lens proteome homeostasis.

This study demonstrated that enhancing the ubiquitinating activity is a valid approach to prevent the accumulation and aggregation of mutant crystallins. Although the results were obtained using V76D mutant γD-crystallin as a model substrate, the impact of this work is not limited to the clearance of mutant γD-crystallins, but also applies to other forms of misfolded or damaged proteins, because many forms of damaged proteins, including truncated, deamidated, and oxidized crystallins, are selectively degraded by the UPP.41,47-49,62,73-76 In summary, this study proved the principle that timely degradation of mutant proteins in living lens cells prevents its accumulation and aggregation. However, these results need to be confirmed in fiber cells in future studies, since the majority of mutant crystallins are in lens fiber cells. We previously demonstrated that lens fiber cells, including fibers in the lens nuclear region, have a functional UPP. Given the similarity of the UPP in lens epithelial cells and lens fibers, it is reasonable to expect that mutant crystallins in lens fibers should also be selectively degraded by the UPP and that enhancement of the degradation capacity in fiber cells would promote the degradation of mutant and other kinds of damaged proteins. However, this hypothesis currently cannot be tested in a cultured lens system, because there is no method available to deliver CHIP or other rate-limiting components of the UPP into fibers of cultured lenses. Future experiments using transgenic mice to overexpress these rate-limiting components in lens fibers could provide direct evidence for the benefits of enhancing UPP capacity in preventing the accumulation and aggregation of mutant and damaged crystallins.

In summary, this study suggests that enhanced ubiquitinating activity can prevent accumulation and aggregation of mutant crystallin by enhancing protein degradation. This should have salutary effects. Conversely, inhibition of the UPP results in the accumulation and aggregation of mutant proteins. Thus, preserving or stimulating the activity of the UPP would be beneficial for lens proteome homeostasis and cataract prevention.

References

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