The inherited retinal degenerations are typified by retinitis pigmentosa (RP), a heterogeneous group of inherited disorders that causes the destruction of photoreceptor cells, the retinal pigmented epithelium, and choroid. This group of blinding conditions affects over 1.5 million persons worldwide. Approximately 30–40% of human autosomal dominant (AD) RP is caused by dominantly inherited missense mutations in the rhodopsin gene. Here we show that P23H, the most frequent RP mutation in American patients, renders rhodopsin extremely prone to form high molecular weight oligomeric species in the cytoplasm of transfected cells. Aggregated P23H accumulates in aggresomes, which are pericentriolar inclusion bodies that require an intact microtubule cytoskeleton to form. Using fluorescence resonance energy transfer (FRET), we observe that P23H aggregates in the cytoplasm even at extremely low expression levels. Our data show that the P23H mutation destabilizes the protein and targets it for degradation by the ubiquitin proteasome system. P23H is stabilized by proteasome inhibitors and by co-expression of a dominant negative form of ubiquitin. We show that expression of P23H, but not wild-type rhodopsin, results in a generalized impairment of the ubiquitin proteasome system, suggesting a mechanism for photoreceptor degeneration that links RP to a broad class of neurodegenerative diseases.

Retinitis pigmentosa (RP) is a heterogeneous group of inherited diseases that cause blindness due to progressive degeneration of rod and cone photoreceptors in the human retina (reviewed in Ref. 1). Approximately 43% of RP is inherited as autosomal dominant (AD) alleles of genes that are expressed predominantly or exclusively in rod photoreceptors. The recessive forms of RP are primarily the result of null mutations eliminating enzymes critical to photoreceptor function. More than 100 distinct mutations in the rhodopsin gene have been documented, accounting for 30–40% of ADRP. The most common abnormality found to cause RP (2) is a single-base substitution in codon 23 (P23H) of the rhodopsin gene, accounting for >7% of all cases of dominant retinitis pigmentosa in the United States (3, 4).

Although the signal transduction cascades leading to caspase activation in RP are unknown (reviewed in Ref. 5), studies in rats and mice expressing rhodopsin transgenes with mutations that are equivalent to those known to cause ADRP in humans suggest that photoreceptor loss is due to apoptotic cell death (6–8). Humans (9) and mice (10, 11) with a single null rhodopsin allele exhibit minimal photoreceptor degeneration, suggesting that ADRP is not the result of haploinsufficiency at the rhodopsin locus.

Studies of mutant rhodopsin molecules expressed heterologously in mammalian cells in culture have suggested that ADRP-linked rhodopsin mutations fall into two classes based on the differing intracellular fates of the mutant proteins. Class I mutants are expressed at wild-type levels in HEK293 or COS cells, where they traffic to the plasma membrane and produce functional photopigment when reconstituted with 11-cis-retinal, suggesting that they are correctly folded (12–14). These mutants, the majority of which are strikingly clustered within the carboxyl-terminal cytoplasmic domain of rhodopsin, appear to be defective in their ability to traffic correctly to rod outer segments (15, 16), possibly because of defective interaction with the retrograde microtubule motor cytoplasmic dynein (17). By contrast, the vast majority of ADRP-causing rhodopsin mutations are class II mutants, which are defective in their ability to fold (18, 19). How rhodopsin misfolding causes ADRP is unknown, although misfolding has recently been suggested to be closely linked to the gain-of-function that underlies disease pathogenesis (19).

When expressed in HEK293 or COS cells, class II mutants fail to acquire complex oligosaccharides indicative of transit through the Golgi apparatus and are severely defective or unable to produce functional photopigment on reconstitution with 11-cis-retinal (12, 14). These findings strongly support the conclusion that class II mutants are unable to fold correctly within the endoplasmic reticulum. This conclusion is also supported by the observation that class II mutants fail to accumulate to high levels in transfected cells (as wild-type rhodopsin), suggesting that they are subject to enhanced intracellular deg-
rhodopsin (12). The mechanism by which class II mutants are degraded, however, is unknown.

Despite an extensive body of data, the mechanisms by which rhodopsin mutations initiate the signaling events leading to photoreceptor death and retinal degeneration remain a mystery. The dominant inheritance pattern, together with the lack of a haploinsufficient phenotype, imply that that rhodopsin-linked ADRP results from a toxic gain of function at the rhodopsin locus. However, the only phenotypic consequence to be attributed to class II mutations in rhodopsin, an inability to fold correctly, is a loss of function.

The discovery that most class II mutants are folding-defective places ADRP within a family of conformopathological diseases that includes most neurodegenerative disorders (20, 21). Like ADRP, the familial forms of these diseases are usually inherited as delayed onset, highly penetrant, dominant traits. In some cases, pathogenesis has been linked to enhanced susceptibility of the mutant gene product to degradation by the ubiquitin proteasome system (22, 23). Degenerating neurons in these diseases accumulate high molecular weight forms of mutant gene product that are usually segregated within intracellular inclusion bodies that are often heavily modified with ubiquitin (Ub) (24–26). By contrast, inclusion bodies and Ub immunoreactivity have not been reported to be prominent histopathological features of degenerating photoreceptors in transgenic animals or in retinas from human RP patients in early stages of the disease (27). Thus, although RP and other central nervous system neurodegenerations appear to share a common etiology, insofar as they are all linked to the production of abundantly expressed, folding-defective polypeptides, they differ in that overt inclusion bodies have not been reported in RP. A simple explanation for this difference might be that the retina may be more effective at eliminating misfolded proteins or preventing the accumulation of aggregated protein products than other central nervous system tissues.

In this study, we have investigated the role of the ubiquitin-proteasome system in the disposal of RP-linked mutant rhodopsin. Our data reveal that the class II rhodopsin mutant, P23H, is a substrate for Ub-dependent degradation by the proteasome. We find that high molecular weight, ubiquitylated forms of P23H accumulate when expressed in cultured cells. We have used fluorescence resonance energy transfer (FRET) to demonstrate that the aggregation of P23H is intracellular, and furthermore, occurs even at very low expression levels, unlike wild-type rhodopsin and the class I mutant, V345M. Finally, our data indicate that aggregation of P23H in cells leads to impairment of the function of the ubiquitin proteasome system, as has recently been demonstrated for other aggregated proteins, including the mutant form of huntingtin linked to Huntington’s disease (28). Therefore, rhodopsin aggregation may represent a toxic gain of function associated with class II mutations in the pathogenesis of ADRP, raising the possibility that retinal degeneration may share a common pathogenic mechanism with other late-onset neurodegenerative diseases of the central nervous system.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Plasmids**—The following antibodies were used in this study: Rhod1D4 (29) (gift from R. Molday, University of British Columbia), B6–30 (gift from P. Hargrave, University of Florida), anti-Hisα (MMS-156P, BabCO/Covance Research Products, Denver, PA), monoclonal α-tubulin (GTU-88, Sigma Chemical Co., St. Louis, MO). The c-myc-Ub plasmids have been described previously (30). Wild-type and V345M (gift from J. Nathans (Johns Hopkins University)). V345M was made by site-directed mutagenesis using QuikChange (Stratagene, La Jolla, CA). YFP and CFP rhodopsin and P23H plasmids have been previously described (31).

**Cell Culture**—Human embryonic kidney 293 (HEK) cells were maintained in Dulbecco’s modified Eagle’s medium and transfected as described previously (30). Transient transfections were carried out by adding plasmid DNA as a calcium phosphate precipitate (32).

**Biological Characterization of Rhodopsin Aggregates**—Cell pellets from transfected and washed HEK cells were lysed in 250 μl of ice-cold buffer A (PBS, pH 7.5, 5 mM EDTA, 1% TX-100) plus protease inhibitor mixture (Roche Molecular Biochemicals). For 30 min on ice, insoluble material was recovered by centrifugation at 13,000 × g for 15 min and solubilized in 50 μl (PBS, 1% SDS) for 10 min at room temperature. After addition of 200 μl of buffer A, samples were sonicated for 20 s with a microtip sonicator.

**Fluorescence Microscopy**—Cells were seeded onto #1 coverslips. For drug treatments, ALLN (5–10 mg/ml, Calbiochem, La Jolla, CA) and nocodazole (10 mg/ml, Sigma Chemical Co.) in Me2SO were added to the culture medium 12 h before fixation. Cells were fixed in −20 °C methanol or 4% paraformaldehyde (20 min). After fixation, cells were washed extensively in PBS and blocked with 10% bovine serum albumin for 10 min and then incubated with primary antibodies for 60 min at room temperature. Cells were washed 5× for 5 min each and incubated with fluorescein-conjugated secondary antibodies at 10 μg/ml final concentration. Cells were washed again (5× for 5 min each) and then incubated for 3 min in PBS plus 10 μg/ml bisbenzimid (Sigma Chemical Co.) to stain DNA. Cells were washed a final time, mounted onto slides in Fluoromount-G (Electron Microscopy Sciences, Fort Washington, PA). For CYP fusion proteins, the cells were fixed in 4% paraformaldehyde. Epifluorescence micrographs were obtained on a Zeiss Axiomvert microscope with a 63× oil objective (numerical aperture, 1.4). Digital (12-bit) images were acquired with a cooled charge-coupled device camera (Trenton Instruments, NJ) using Metamorph software (Universal Imaging, Media, PA). The excitation filters used were: 355DF20 (DAPI), 440 DF20 (CFP), 490DF10 (YFP and FITC), and 570DF10 (Texas red). Emission filters were: 460DF20 (DAPI), 475 DF20 (CFP), 535DF25 (FITC and YFP), and 630 DF20 (Texas red). The dichroic measurements were: 420 DCLP (DAPI), 445DCLP (CFP), 505 DCLP (FITC and YFP), and 595DCLP (Texas red).

**FRET Measurements**—FRET was determined as previously described (31). Fluorescence spectra were recorded on suspended cells (106 cells/ml) in a Spex Fluorolog fluorometer with a Spex 1620 dual grating emission monochromator (Spex Industries, Metuchen, NJ). FRET measurements were made by exciting the donor (CFP) at 425 nm and monitoring the emission spectrum between 450 and 600 nm. All FRET spectra were corrected for background YFP fluorescence using a non-FRET pair consisting of identical amounts of YFP fusion construct and unlabeled rhodopsin (wild-type or mutant). To quantify FRET, the ratio of fluorescence at 525 nm (YFP) to the fluorescence at 476 nm (CFP) was measured. For P23H-CFP alone, this ratio was found to be 0.42 ± 0.013 (S.E., n = 5). This value was subtracted from the ratio obtained for any given sample to yield the "FRET value." IntrinsYFP fluorescence was recorded by excitation at 490 nm and emission between 505 and 600 nm. Slit widths were 2 nm for all experiments.

**Ubiquitin Proteasome System Activity**—GFP−1 cells (28) were transiently transfected using the calcium phosphate method. Cells were harvested by trypsinization 48 h post-transfection and fixed in 4% formaldehyde in PBS for 30 min at room temperature. Cells were permeabilized in PBS, 0.1% Triton X-100, and 2% bovine serum albumin and stained in suspension using the indicated antibody at room temperature for 1 h followed by phycocerythrin-conjugated secondary antibody (1:500) for 1 h at room temperature. GFP and phycocerythrin intensities were simultaneously measured on 50,000 cells for each sample using an XLI analyzer ( Coulter). Histograms were generated using FlowJo software (Tree Star, Inc.).
**RESULTS**

**P23H Rhodopsin Is Prone to Aggregate**—Aggregation of wild-type rhodopsin and the RP-linked mutants P23H and V345M was assessed by SDS-PAGE immunoblot analysis of detergent-soluble and detergent-insoluble extracts from HEK293 cells transiently expressing these constructs at varying levels (Fig. 1A). At low expression levels (<0.5 µg), wild-type rhodopsin migrated predominantly as a detergent-soluble, diffuse band at a molecular mass of ~40–43 kDa. This species corresponds to monomeric, mature rhodopsin containing complex N-linked glycans, as evidenced by its mobility and by its sensitivity to cleavage by protein:N-glycanase (PNGase) but not by endoglycosidase H (Fig. 1B). Endoglycosidase H is specific for high mannose N-linked oligosaccharide structures typical of proteins that have not matured beyond the endoplasmic reticulum, whereas PNGase cleaves all N-linked glycans. At higher

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**Fig. 1.** High molecular weight complexes and intracellular processing of ADRP-linked rhodopsin mutants in HEK293 cells. A. Formation of high molecular weight oligomers is a function of expression and genotype. Cells were transiently transfected with the indicated amount of plasmid-encoding wild-type (WT) rhodopsin or the ADRP mutants P23H (class II) or V345M (class I), separated into detergent-soluble (upper panels) or insoluble fractions (lower panels), and subjected to immunoblot analysis with a rhodopsin (1D4) mAb. Control cells (lane v) were transfected with vector alone. Some cells were incubated in the presence of the proteasome inhibitor ALLN as indicated. B, glycosylation status of mutant and wild-type rhodopsin species. Detergent-soluble and -insoluble fractions of lysates from HEK293 cells transfected with 1 µg of plasmid were subjected to digestion with endoglycosidases H or PNGase, as indicated, prior to immunoblot analysis. The data in this analysis are representative of at least three independent experiments.
expression levels of wild-type rhodopsin (Fig. 1A), additional high molecular weight species, suggestive of SDS-resistant multimers, were also detected. All of the monomeric rhodopsin partitioned into the detergent-soluble fraction, whereas the slower migrating forms partitioned into both detergent-soluble and -insoluble fractions. These data, together with the results
FIG. 3. Rhodopsin expression and spontaneous inclusion body formation in the absence of proteasome inhibitors. A–C, fusion of YFP to rhodopsin does not alter its trafficking. HEK cells were transfected with 0.5 μg of wild-type-YFP (A), V345M-YFP (B), or P23H-YFP (C) and fixed 48 h later, and visualized for YFP (green). DNA was stained (blue) with bis-benzimide. The arrow in C indicates a spontaneous P23H Rhodopsin Aggregation and the Ubiquitin Proteasome Pathway
from immunofluorescence studies (Figs. 2 and 3), are in agreement with previous studies (12), which concluded that the majority of rhodopsin, expressed in HEK293 cells, folds into a native conformation. At high levels of expression, a minor fraction of wild-type rhodopsin forms high molecular weight complexes that are insoluble in SDS and non-ionic detergent, suggesting that the capacity of these cells to fold rhodopsin is limited.

P23H differed markedly from wild-type rhodopsin both in terms of mobility and detergent solubility. The small amounts of monomeric P23H detected migrated with a slightly faster mobility than that of monomeric wild-type rhodopsin and were sensitive to cleavage by endoglycosidase H (Fig. 1B), indicating that they were retained within the ER. The majority of P23H, however, migrated as dimers and higher molecular weight oligomers. These oligomers are evidence of a non-native conformation, because rhodopsin is monomeric in its native membrane (33). A substantial fraction of P23H partitioned into the detergent-insoluble fraction, suggesting that P23H is more prone to aggregate than wild-type rhodopsin (Fig. 1). In contrast to P23H, both the mobility and the detergent solubility behavior of the class I mutant, V345M, were indistinguishable from those of wild-type rhodopsin (Fig. 1, A and B).

Exposure of cells expressing low levels of wild-type or V345M rhodopsin to the proteasome inhibitor (ALLN) resulted in a substantial increase in the abundance of all electrophoretic rhodopsin species (Fig. 1A), suggesting that some folding-competent rhodopsin molecules are degraded by the proteasome in HEK cells. Indistinguishable results were obtained in cells exposed to the more specific inhibitors MG132 (34) and lactacystin (35) (data not shown). In contrast, exposure of P23H-expressing cells to ALLN increased the proportion of oligomeric P23H species but failed to promote the formation of mature monomer, suggesting that proteasomes also participate in the degradation of this folding-incompetent class II mutant rhodopsin.

Together, these data confirm that, in HEK cells, wild-type rhodopsin and a class I mutant (V345M) are able to fold and mature beyond the ER, whereas the class II mutant P23H is unable to fold productively. Moreover, our data suggest that P23H is considerably more prone to forming non-native oligomers than is wild-type or V345M.

Misfolded Rhodopsin Accumulates in Aggresomes—We used immunofluorescence microscopy to determine the intracellular localization of wild-type and mutant rhodopsins. As shown in Fig. 2A, wild-type and V345M were present in a predominantly plasma membrane distribution, consistent with previous reports and with our observation (Fig. 1) that wild-type and V345M rhodopsin are detergent soluble and modified by complex oligosaccharides. In contrast, under the same conditions, P23H was present in a predominantly ER distribution (Fig. 2A), consistent with its designation as a class II mutant and with data indicating that it remains core-glycosylated (Fig. 1B).

To assess the role of microtubules in the formation of P23H inclusion bodies, cells expressing low levels of P23H were treated simultaneously with ALLN to induce accumulation of aggregates and the microtubule-destabilizing agent, nocodazole (Fig. 2B, right-hand panel). In contrast to cells treated with ALLN alone (Fig. 2B, left panel), cells treated with ALLN and nocodazole together had numerous intense, small foci of P23H that were diffusely distributed throughout the cytoplasm. However, the extent of rhodopsin aggregation, as assessed by detergent solubility and electrophoretic mobility (Fig. 2D) and by fluorescence resonance energy transfer (31), was unaffected by nocodazole treatment. These data demonstrate that protein aggregation and inclusion body formation are distinct, separable processes and that delivery of misfolded mutant or wild-type rhodopsin to cytoplasmic inclusion bodies requires an intact microtubule cytoskeleton. A direct inference from this observation is that the absence of rhodopsin-containing inclusion bodies, as in retinas from human ARDP and animal models thereof, does not necessarily rule out a role for rhodopsin aggregation in pathogenesis.

Aggregation Is a Gain of Function Linked to the P23H Mutation—To elucidate the relationship between protein expression and aggregation, we examined the cellular distribution and steady-state levels of P23H, V345M, and wild-type rhodopsin in HEK cells transfected with varying levels of plasmid (Figs. 3 and 4). To study the intrinsic aggregation properties of these proteins, these studies were conducted in the absence of any added proteasome inhibitors. We used fusion proteins between the C terminus of wild-type, V345M, or P23H rhodopsin with GFP or the spectrally shifted variants cyan (CFP) or yellow (YFP) fluorescent protein (31). As shown in Fig. 3 (A–C), the appended fluorescent proteins did not influence the intracellular distribution of the rhodopsins studied. Wild-type rhodopsin and V345M were predominantly localized at the plasma membrane, whereas the majority of P23H exhibited a diffuse, ER-like distribution. Cells expressing wild-type or V345M rhodopsin fusions were 4- to 5-fold brighter than those expressing P23H, consistent with the suggestion (12–14) that the P23H mutation destabilizes rhodopsin (Fig. 3E). At low expression levels (0.5 μg), inclusion bodies were found only in rare (∼1%) cells expressing wild-type rhodopsin-YFP or V345M-YFP, but more frequently (∼3%) in cells transfected with equal amounts of P23H-YFP plasmid (Fig. 3D, left). At higher transfection levels, the fraction of cells with inclusion bodies increased for both wild-type and P23H rhodopsin fusions (Fig. 3D, right). This reveals that rhodopsin inclusion bodies can form in the absence of proteasome inhibitor. However, the increased tendency of P23H-YFP to aggregate (compared with wild-type) is dramatically illustrated when the fraction of cells with inclusion bodies is normalized to the total rhodopsin fluorescence. Total rhodopsin expression was quantified by monitoring the intrinsic CFP or YFP fluorescence, because the absolute fluorescence is not significantly influenced by subcellular localization.
FIG. 4. Aggregation is a gain of function for P23H. A, fluorescence resonance energy transfer was used to study rhodopsin aggregation in the absence of proteasome inhibitor. The solid line represents cells singly transfected with P23H-CFP mixed with cells singly transfected with P23H-YFP. The dashed line represents cells that were co-transfected with P23H-CFP and P23H-YFP and mixed with mock transfected cells. The spectra were corrected for cellular autofluorescence and background YFP fluorescence. B, relationship between extent of wild-type (open squares) or P23H (closed squares) rhodopsin aggregation (determined by FRET) and total rhodopsin expression (assessed by CFP fluorescence). C, a comparison of FRET in wild-type, P23H, and V345M at low and high protein expression levels (mean of n = 5 observations). Protein expression was quantified by intrinsic YFP fluorescence and divided into two regimes of low (<5 × 10^5 counts per second) and high (>10^6 counts per second) YFP fluorescence. All experiments had equal amounts of CFP and YFP plasmids. D, relationship between the fraction of oligomeric wild-type (open squares) or P23H (closed squares) rhodopsin (determined from the immunoblot in Fig. 1A) and DNA used for transfection.

We have recently reported that FRET is a highly sensitive method to monitor, in vivo, the aggregation of misfolded proteins (31). FRET results from the transfer of energy from a fluorescent donor (CFP) in its excited state to another excitable moiety, the acceptor (YFP), via non-radiative dipole-dipole interactions. To establish that P23H aggregation occurs intracellularly and is not an artifact of cellular lysis or proteasomal inhibition, we used FRET between rhodopsin C/YFP fusions to monitor rhodopsin aggregation in living cells, in the absence of drugs. The fluorescence emission spectrum obtained from a mixture of HEK cells singly transfected with either P23H-CFP or P23H-YFP revealed distinct peaks at 476 and 505 nm, corresponding to CFP emission (Fig. 4A, solid line). In contrast, the spectrum of cells co-transfected with both plasmids revealed a reduction in the CFP emission peaks and an appearance of a shoulder at 525 nm, corresponding to the sensitized emission from YFP as a result of FRET. We then monitored FRET (using a FRET scale based on the ratio of fluorescence emission at 525–476 nm (31)) in cells expressing wild-type, V345M, and P23H as a function of total rhodopsin expression level. Total rhodopsin levels were quantified either by the intrinsic CFP (Fig. 4B) or YFP (Fig. 4C) fluorescence emission. At low expression levels, FRET was undetectable in cells expressing wild-type rhodopsin-C/YFP, despite a robust fluorescence signal (Fig. 4B). The FRET value increased with increasing wild-type rhodopsin expression, reaching a plateau at a level of ~0.4. In contrast, in cells expressing P23H, FRET was essentially maximal at the same plateau as the wild-type protein, even at the lowest levels of expression. The aggregation behavior of V345M, assessed using FRET (Fig. 4C), is similar to that of wild-type rhodopsin.

Remarkably, the aggregation behavior of wild-type and P23H rhodopsin, as assessed by FRET, correlates extremely well with the extent of association of these molecules into SDS-insoluble native oligomers (Figs. 1A and 4D). Together these results confirm that P23H is more aggregation-prone than wild-type rhodopsin and further demonstrate that the propensity to aggregate is a gain of function attributable to the P23H mutation.

Misfolded Rhodopsin Is Degraded by the Ubiquitin-Proteasome System—The increased steady-state level of mutant and wild-type rhodopsin induced by exposure of cells to proteasome inhibitors (Fig. 1A) suggests a role for proteasome-dependent proteolysis in rhodopsin turnover. Because degradation of misfolded integral membrane proteins from the ER requires a functional Ub pathway (30, 37), we used two different approaches to evaluate the role of Ub in the degradation of mutant and wild-type rhodopsin expressed in HEK cells (Fig. 5). In the first approach, we used immunopurification to show that P23H is ubiquitinylated to a far greater extent than is wild-type rhodopsin. Cells were transfected with rhodopsin or P23H.
Rhodopsin Aggregation and the Ubiquitin Proteasome Pathway

Protein degradation by the proteasome is strongly enhanced by attachment of multiple Ub moieties in a polymer formed by isopeptide linkages between the carboxyl terminus of one Ub and the ε-amino group of Lys-48 of another Ub (38–40). Mutations at Lys-48 can function as chain-terminating dominant negative modulators of proteolysis (38). To confirm a role for Ub in rhodopsin and P23H turnover, cells were co-transfected with the respective rhodopsin plasmid together with excess wild-type or K48R-Ub plasmid (or empty vector control). The steady-state levels of both wild-type and P23H were substantially increased by co-expression of K48R-Ub when compared with vector co-transfection. This strongly supports the conclusion that the turnover of both forms is Ub-dependent (Fig. 5B). As with the proteasome inhibitors, K48R-Ub co-expression increased rhodopsin levels in both detergent-soluble and -insoluble fractions, indicating that undegraded rhodopsin molecules aggregate. Interestingly, co-expression of wild-type Ub led to a reproducible and significant decrease in steady-state levels of the rhodopsin proteins, suggesting that, under these experimental conditions, the levels of free Ub may be rate-limiting for degradation.

**P23H Aggregation Leads to Impairment of the Ubiquitin-Proteasome Pathway**—Recently we reported that cytoplasmic aggregation of a mutant form of cystic fibrosis transmembrane conductance regulator (ΔF508-CFTR) and a mutant form of huntingtin (Q103) causes a decrease in the function of the ubiquitin-proteasome system (UPS) (28). We developed a novel reporter (GFPu) of UPS activity consisting of GFP conjugated to a Ub-specific degron (28). Because GFPu is a rapidly degraded UPS substrate, impaired UPS function leads to increased steady-state GFPu concentration, which can be monitored as a change in mean fluorescence. To investigate the cellular consequences of P23H aggregation, we measured the fluorescence of GFPu-1, an HEK293 line harboring stable expression of ΔF508-CFTR, an integral membrane protein, which we have previously shown to be extremely aggregation-prone (36). Transfected cells were immunostained for rhodopsin (or Q25 and CFTR), and the levels of the transfected protein and GFPu were analyzed simultaneously by two-color flow cytometry. GFPu levels in the cells expressing low or high levels of transfected protein are represented as normalized population histograms (Fig. 6). The control experiments (Fig. 6, A and B) confirmed our previous finding that GFPu fluorescence was increased by expression of ΔF508 CFTR but not by expression of Q25. Similarly, there was no detectable increase in GFPu fluorescence in cells expressing wild-type rhodopsin (Fig. 6C) even at levels up to 4 μg. However, expression of P23H resulted in a significant and reproducible increase in GFPu fluorescence that was evident even at expression levels below those used for wild-type rhodopsin. Protein degradation by the proteasome is strongly enhanced by attachment of multiple Ub moieties in a polymer formed by isopeptide linkages between the carboxyl terminus of one Ub and the ε-amino group of Lys-48 of another Ub (38–40). Mutations at Lys-48 can function as chain-terminating dominant negative modulators of proteolysis (38). To confirm a role for Ub in rhodopsin and P23H turnover, cells were co-transfected with the respective rhodopsin plasmid together with excess wild-type or K48R-Ub plasmid (or empty vector control). The steady-state levels of both wild-type and P23H were substantially increased by co-expression of K48R-Ub when compared with vector co-transfection. This strongly supports the conclusion that the turnover of both forms is Ub-dependent (Fig. 5B). As with the proteasome inhibitors, K48R-Ub co-expression increased rhodopsin levels in both detergent-soluble and -insoluble fractions, indicating that undegraded rhodopsin molecules aggregate. Interestingly, co-expression of wild-type Ub led to a reproducible and significant decrease in steady-state levels of the rhodopsin proteins, suggesting that, under these experimental conditions, the levels of free Ub may be rate-limiting for degradation.

**DISCUSSION**

Accumulation of aggregated, multiubiquitinated proteins within intracellular inclusion bodies in affected neurons has long been recognized as a hallmark of most neurodegenerative diseases, suggesting that these diverse disorders may be related through a common pathogenic mechanism linked to protein misfolding and aggregation. The presence of highly ele-
vated levels of multiubiquitinylated protein within inclusion bodies in affected neurons in most sporadic and inherited neurodegenerative diseases (24–26), together with recent biochemical (28) and genetic studies (41–45), suggests that dysfunction of the ubiquitin proteasome pathway (UPS) is also intimately tied to the underlying cellular pathogenesis. The data presented in this study reveal that a mutation linked to ADRP, a leading cause of adult onset blindness, results in the production of a misfolded and highly aggregation-prone form of rhodopsin that in cultured cells is both a substrate and an inhibitor of the UPS.

These data suggest that UPS impairment may contribute to ADRP pathogenesis and, therefore, that retinal degeneration may share hitherto unsuspected common pathogenic features with other adult onset degenerative diseases of the central nervous system. Class II mutants like P23H fail to acquire

**Fig. 6. Aggregation of P23H rhodopsin impairs the function of the Ub-proteasome system.** GFP⁺ cells (harboring the GFP⁺ UPS reporter) were transfected with 4 μg of plasmid encoding huntingtin Q25-myc (A), ΔF508 CFTR (B), wild-type rhodopsin (C), or P23H (D). Transfected cells were fixed, immunostained with c-myc antibody (A), CFTR antibody (B), or rhodopsin (1D4) mAb (C and D) and analyzed simultaneously by flow cytometry for expression of the respective antigens and GFP⁺ fluorescence. Traces shown are histograms (normalized to total cell number) of GFP⁺ fluorescence in cells containing high (dashed line) or low (solid line) levels of each transgene.
complex oligosaccharides and are not able to bind 11-cis-retinal to form a rhodopsin chromophore, suggesting that class II ADRP, like many other dominantly inherited neurodegenerative diseases, is associated with defective protein folding (12–14). Our finding that P23H is ubiquitinylated and is a substrate for the UPS, reveals a second feature common to ADRP and other neurodegenerative diseases such as familial amyotrophic lateral sclerosis (FALS), in which disease-causing mutations enhance the susceptibility of the affected protein (copper and zinc superoxide dismutase (SOD)) to degradation by the UPS (23, 46, 47). Finally, the data in the present study show that the P23H mutation renders rhodopsin highly prone to aggregation, revealing a third feature common between ADRP and nearly all other neurodegenerative diseases.

In this study, three lines of evidence, formation of high molecular weight SDS-resistant oligomers, enhanced sequestration of P23H in aggresomes and FRET, all support the conclusion that P23H is significantly more prone to aggregation than is wild-type rhodopsin. Strikingly, unlike other aggregation-prone proteins, like SOD, CFTR, and mutant huntingtin, in which we find a mixture of aggregated and non-aggregated forms at steady-state, P23H is remarkable in that aggregates are the predominant form detectable in cells at all expression levels. Whether this property results from the extremely hydrophobic nature of rhodopsin or an increased propensity to form β-sheet structures, which underline the aggregates of many other proteins (48), will require further investigation.

It is essential to distinguish between aggregates, which can be defined as non-native protein oligomers, and inclusion bodies, which are microscopically distinct cellular regions into which aggregated proteins are sequestered (26). For example, formation of inclusion bodies composed of aggregated mutant SOD is a relatively late event in the progression of spinal motor neuron disease in a transgenic mouse model of FALS, occurring several months after the onset of detectable cellular pathology (46, 49). In contrast to inclusion bodies, formation of biochemically distinct, detergent-insoluble, SOD oligomers and high molecular weight aggregates precedes by several months the appearance of inclusion bodies and coincides with the earliest manifestations of cellular pathology (23). These findings suggest that it is the adoption of a non-native oligomeric conformation, i.e. aggregation, and not inclusion body formation, that underlies FALS and perhaps other aggregation-linked neurodegenerative diseases. The oligomeric forms of rhodopsin, much like those of SOD, are non-native structures, given the fact that the wild-type is monomeric (33). Thus, they fit our definition of aggregates, being "non-native oligomers."

One possible explanation for the apparent absence of inclusion bodies in photoreceptors from degenerating retina is that photoreceptors may be more susceptible to the toxicity of protein aggregates than are other central nervous system neurons or transfected HEK cells. Microtubules in photoreceptors are organized around the basal bodies within the specialized non-motile connecting cilium at the junction between the light sensitive outer segment and the metabolically and biosynthetically active inner segment (50, 51). Perhaps this highly specialized microtubule array organization limits the capacity of photoreceptors to clear protein aggregates by the type of dynein-mediated processes, which operate in non-ciliated cells to concentrate aggregated proteins in pericentriolar inclusion bodies (26). Lacking such a mechanism to sequester toxic P23H aggregates into inclusion bodies could lower the threshold for aggregate toxicity and cause photoreceptors to die without the formation of readily detectable foci.

It is also possible that the absence of detectable inclusion bodies within photoreceptors in ADRP reflects an increased capacity of the retina to eliminate damaged cells compared with other central nervous system regions. Lysis of the photoreceptor inner segment is a prominent and early feature of ADRP pathogenesis, suggesting that cells with the highest aggregate burden sufficient to produce detectable inclusion bodies may be eliminated preferentially. Because of the laminar organization of the retina and the close proximity of the retinal pigmented epithelium, a layer of highly phagocytic cells contacting the photoreceptor outer segments, it is likely that removal of moribund neurons or fragments thereof is more efficient in retina than in other regions of the brain. In *Drosophila*, mutations affecting rhodopsin folding also cause photoreceptor degeneration (52). However, in flies, which lack the equivalent of a retinal pigmented epithelium, expression of folding-defective rhodopsin mutants does lead to the formation of cytoplasmic inclusion bodies and a generalized disruption of internal membranes (53). These observations confirm that aggregation into cytoplasmic inclusion bodies is a property intrinsic to folding-defective mutant forms of rhodopsin and suggest that the mammalian retina may possess specialized mechanisms to prevent their accumulation. Our observation that P23H-expressing cells that have negligible inclusion bodies (<3%) still have maximal FRET ties in very well with the lack of observable inclusion bodies in ADRP. It suggests that aggregates, and their potential toxicity, can persist in the absence of observable inclusion bodies.

Although it is formally possible that the high molecular weight rhodopsin and P23H species observed in this study are post-lysis artifacts generated during sample preparation, three lines of evidence argue strongly that these species are generated intracellularly. First, the observation of aggresomes in cells expressing P23H strongly indicates that P23H aggregation occurs in vivo. Aggresome formation by P23H does not require proteasome inhibitors, because they are observed in cells in the absence of any drug treatment. Second, because FRET is efficient only for C/YFP fluorophores that are within 100 Å of each other (54), our data suggest that the P23H mutation endows the rhodopsin molecule, normally a monomer even at the high concentrations present within the rod outer segments (39), with the ability to self-associate into compact structures. Finally, we observe identical patterns of electrophoretic mobilities of P23H rhodopsin when the cells are lysed in the presence of alkylating agents such as iodoacetamide, which prevent the formation of non-native disulfide-linked species in the highly oxidizing environment of SDS-PAGE (55).

The data presented here demonstrate that an increased propensity to self-associate into high molecular weight aggregates is a robust gain of function, which is directly linked to pathogenesis caused by a class II ADRP mutant rhodopsin. One way in which this property might be cytotoxic is by interfering with the function of the UPS, a proteolytic system that plays a crucial role as a cellular "master regulator" in all eukaryotic cells (56). Whether or not rhodopsin aggregation and UPS impairment are general features of other class II mutants must await future studies. Nevertheless, our data suggest a novel link between ADRP and other hereditary neuropathies and suggest that the consequences of protein aggregation may have a more general role in neurodegeneration than hitherto anticipated.

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