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Molecular Defects of the Disease-Causing Human Arrestin-1 C147F Mutant

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Citation: Vishnivetskiy SA, Sullivan LS, Bowne SJ, Daiger SP, Gurevich EV. Purpose. The purpose of this study was to identify the molecular defect in the disease-causing human arrestin-1 C147F mutant.

METHODS. The binding of wild-type (WT) human arrestin-1 and several mutants with substitutions in position 147 (including C147F, which causes dominant retinitis pigmentosa in humans) to phosphorylated and unphosphorylated light-activated rhodopsin was determined. Thermal stability of WT and mutant human arrestin-1, as well as unfolded protein response in 661W cells, were also evaluated.

RESULTS. WT human arrestin-1 was selective for phosphorylated light-activated rhodopsin. Substitutions of Cys-147 with smaller side chain residues, Ala or Val, did not substantially affect binding selectivity, whereas residues with bulky side chains in the position 147 (Ile, Leu, and disease-causing Phe) greatly increased the binding to unphosphorylated rhodopsin. Functional survival of mutant proteins with bulky substitutions at physiological and elevated temperature was also compromised. C147F mutant induced unfolded protein response in cultured cells.

CONCLUSIONS. Bulky Phe substitution of Cys-147 in human arrestin-1 likely causes rod degeneration due to reduced stability of the protein, which induces unfolded protein response in expressing cells.

Keywords: arrestin, retinitis pigmentosa, photoreceptors, protein stability

Visual arrestin-1 was first discovered for its ability to bind light-activated phosphorylated rhodopsin (P-Rh*) and quench rhodopsin signaling,2 apparently due to direct competition of active rhodopsin with visual G protein transducin.3,4 The function of arrestin in the termination of rhodopsin signaling is critical: the lack of arrestin,5 the absence of rhodopsin kinase,6,7 the absence8,9 or insufficient number10 of phosphorylation sites in rhodopsin preclude high-affinity arrestin-1 binding11,12 as well as cause visual defects in mice and humans. However, a previously identified loss-of-function mutation in arrestin-113 is recessive, as the other perfectly normal allele is sufficient for function, in line with our earlier finding that as little as 4% of wild-type (WT) arrestin expression level is sufficient for maintaining rod health in mice.14 It is interesting that recently identified C147F mutation in the human arrestin-1 is dominant and causes retinitis pigmentosa.15 Thus, loss of function cannot explain the mechanism of action of this mutant arrestin-1. Cys147 in the human arrestin-1 is homologous to the Cys145 of the bovine protein, which in crystal structures16,17 is placed in the closely packed area in the central crest of the two-domain arrestin-1 molecule between receptor-binding “finger” loop and “139-loop.”18,22 Thus, this mutation can potentially affect at least two aspects of arrestin-1 function, rhodopsin binding and folding. Here we set out to determine the molecular defects of the C147F mutant that might underlie human disease.

METHODS

Materials

[y-32P]ATP, [14C]leucine, and [3H]leucine were from Perkin-Elmer (Waltham, MA, USA). All restriction and DNA-modifying enzymes were from New England Biolabs (Ipswich, MA, USA). Rabbit reticulocyte lysate was from Ambion (Austin, TX, USA), and SP6 RNA polymerase was prepared as described.25

Mutagenesis and Plasmid Construction

For in vitro transcription, the human arrestin-1 cDNA was subcloned into pGEM2 (Promega, Madison, WI, USA) with “idealized” 5′-UTR between Ncol and HindIII sites, as described for the bovine11 and mouse arrestin-1.24 All mutations were introduced in the introduction construct by PCR and verified by dye-deoxy sequencing. For Escherichia coli expression, cDNA encoding WT human arrestin-1 and its C147F mutant was subcloned into pTrcHisB vector between Ncol and HindIII sites (this eliminates His6-tag so that unmodified protein is expressed), as described.25 Test expression in 3 ml bacterial culture, cell lysis, and separation of soluble protein from cell debris were performed, as described.26 In vitro transcription, translation, preparation of phosphorylated and unphosphorylated rhodopsin were performed as described recently.27,28
Electrophoresis, Autoradiography, and Western Blotting

Cell-free translated protein labeled with $^{14}$C-Leu and $^{3}$H-Leu was subjected to SDS-PAGE in 10% polyacrylamide (PAAG) gel, which was then stained with a reagent (GelCode Blue Coomassie-based; Thermo Scientific, City State, USA), de-stained with water, and soaked in 10% 2,4-dihydroxyphenoxazol (PPO) in acetic acid. PPO was then precipitated within the gel by immersing it in water, and radioactive bands were visualized by autoradiography with X-ray film. Aliquots of the lysates of arrestin-expressing E. coli and supermutants after high-speed centrifugation of these lysates containing soluble proteins were subjected to SDS-PAGE in 10% PAAG gel and transferred to polyvinylidene fluoride (PVDF) membranes. Arrestin bands were visualized using pan-arrestin rabbit F431 primary and horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies, followed by incubation with reagent (WestPico ECL, Thermo Scientific) and exposure to X-ray film.

Direct binding assay was performed, as described. 27 Briefly, 1 nM arrestin-1 (50 fmol) was incubated with 0.3 μg P-Rh* or Rh* (11 pmol, yielding final concentration of 0.22 μM) in 50 μL of 50 mM Tris- HCL, pH 7.4; 100 mM potassium acetate; 1 mM EDTA; 1 mM dithiothreitol for 5 minutes at 37°C (or other indicated temperature) under room light. Samples were cooled on ice, whereupon bound and free arrestin-1 were separated at 4°C by gel filtration on a 2-ML column of Sepharose 2B-CL. Arrestin-1 eluting with rhodopsin-containing membranes was quantified by liquid scintillation counting. Nonspecific binding, determined in samples where rhodopsin was omitted, was subtracted.

In Vitro Arrestin Stability Assay

Translated radiolabeled arrestin-1 was incubated for 1 or 2 hours at 42°C, or for up to 24 hours at 37°C, and cooled on ice. The binding of arrestin-1 to P-Rh* in these samples was compared to that of control sample kept on ice, as described above; 1 nM arrestin-1 (50 fmol per sample) was used, as in the standard direct binding assay.

Cell Culture

Photoreceptor-derived 661W 29 cells, 29 which are more appropriate for studies of visual arrestin-1 than most cultured cell lines, were used to express WT human arrestin-1 and its C147F mutant. These cells were originally isolated from a transgenic mouse line expressing SV40 T-antigen under control of human interphotoreceptor retinol-binding protein promoter. 29 The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The protein was precipitated by the addition of nine volumes of methanol and pelleted by centrifugation for 10 minutes at 13,000 rpm (Eppendorf MiniSpin centrifuge). The pellet was washed with 1 mL 90% methanol, dried, and dissolved in SDS sample buffer (Sigma-Aldrich Corp., St. Louis, MO, USA) at 0.5 μg/μL.

Western Blotting

The protein was subjected to SDS-PAGE in 10% (GAPDH and arrestin) or 8% (GRP78/BiP) acrylamide gel and transferred to PVDF membrane. The proteins were visualized using the following primary antibodies: anti-GAPDH (loading control, mouse, 1:1000), anti-arrestin (F431 rabbit polyclonal, 26 1:5000), and anti-BIP (rabbit polyclonal, 1:1000 dilution; Cell Signaling, Danvers, MA, USA). Blots were incubated with primary antibodies overnight at 4°C, washed three times with TBS-0.1% Tween-20, then incubated with corresponding HRP-conjugated secondary antibodies (1:5000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). The bands were developed using ECL pico reagent (Thermo Fisher Scientific) (5 minutes) and visualized by exposure to X-ray film (VWR, Radnor, PA, USA). The signal was quantified using VersaDoc with QuantityOne software (Bio-Rad).

Statistical Analysis

Statistical analysis was performed by ANOVA using analysis software (StatView SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

 Arrestin-1, 16,17 as well as all other vertebrate arrestin isoforms, 30–33 is an elongated two-domain molecule with relatively few contacts between domains (Figs. 1A, 1B), perfectly suited for proposed global conformational change upon receptor binding. 34,35 which was recently confirmed by the structure of the arrestin-1-rhodopsin complex. 19,36 Cys-145 in bovine arrestin-1, homologous to Cys-147 in human protein, is not exposed; it is located between the two loops in the central crest of the receptor-binding arrestin surface (Figs. 1A, 1B), where in the bovine protein it is closely packed with several hydrophobic partners: Leu132, Val247, Tyr255, and Ile323 (Fig. 1C). 17 Thus, the substitution of a relatively small cysteine with a bulky Phe could potentially lead to misfolding and/or temperature-sensitive unfolding of the protein. There are two differences between Cys and Phe: the presence of a functional SH group in the former, but not in the latter, and the size of the side chain. As the vertebrate visual arrestin-1 was not reported to undergo posttranslational modifications, 37 where the SH group could have played a role, we focused on the size of the side chain and generated mutants containing small Ala, medium-sized Val, and bulky Leu, Ile, and Phe residues in position 147. In case of the bovine arrestin, we previously found that Ala or Val in position 143 do not significantly change functional characteristics of WT arrestin-1. 18,38–40

Proper folding is necessary for protein functionality. Protein yield in cell-free translation and the fraction of translated protein that remains soluble after high-speed centrifugation are good indicators of proper folding. 41 Cell-free translation is a relatively demanding system, as the cytoplasm of rabbit reticulocytes is diluted several-fold 42 so that the concentrations of all chaperones that assist protein folding are significantly reduced. Thus, first we compared the yields of WT and mutant forms of the human arrestin-1 in cell-free translation as well as the fraction of soluble arrestin after centrifugation at ~600,000g for 1 hour (Figs. 2A, 2B). We did not detect greater
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FIGURE 1. The localization of cysteine-147 in the arrestin-1 protein. (A, B) Crystal structure of the bovine arrestin-1 (PDB 1CF1) colored by secondary structure: β-strands, blue; β-turns, green; α-helices, red. Cys-147 (Cys-143 in bovine arrestin-1) is blue and indicated. A view from the side (A) and receptor-binding side (B) of the molecule is shown. Note that Cys-147 is located between the two loops in the central crest of the receptor-binding side of the molecule. The both finger loop and 139-loop were implicated in receptor binding by site-directed spin labeling/EPR,18,20 mutagenesis,22 and crystallography.19,21,36 (C) Blow-up of the view from the receptor-binding side showing bovine Cys-143 and its immediate neighbors, Leu-132, Val-247, Tyr-255, and Ile-323 in the bovine arrestin-1, all of which are conserved in the human protein. Note the close packing of Cys-143, suggesting that a much larger side chain of phenylalanine in this position would not fit and therefore would likely cause a shift of neighboring residues and elements containing them.

than 33% difference in the expression levels of WT and mutant forms of the human arrestin-1. These data excluded gross misfolding as the likely reason for pathology caused by the C147F mutation in human patients.15 In all cases, the band with the expected molecular weight of human arrestin-1 contained >95% of radiolabeled protein (Fig. 2B), indicating that WT and mutant forms of human arrestin-1 did not undergo proteolysis.

WT visual arrestin-1 from different species (bovine, mouse, and human) successfully folds even in E. coli, which has fewer chaperones than eukaryotic cells and can be purified from expressing bacteria in large quantities.25,26 Therefore, next we compared the expression of human arrestin-1 C147F mutant with the WT human and bovine proteins in E. coli, as well as the soluble fraction of these proteins (Fig. 2C). To this end, we performed test expression in 3 mL of bacterial culture. After 4-hour induction with isopropyl β-D-thiogalactopyranoside at 30°C, the cells were pelleted and lysed following standard procedure,29 then cell debris along with inclusion bodies (where bacteria place misfolded or denatured proteins) and aggregates were separated from soluble proteins by centrifugation at ~600,000g for 1 hour. Western blotting of the aliquots of cell lysates and supernatants with rabbit pan-arrestin F431 antibody26 revealed that C147F mutant expresses at a higher level than does WT human arrestin-1, but practically all of it is insoluble in contrast to WT bovine and human arrestin-1 (Fig. 2C). Thus, C147F mutant either does not fold as easily as WT protein in the absence of mammalian chaperones or readily denatures and aggregates. Only a small fraction of immunoreactive material was present as smaller molecular weight species, which were visible in all cases (Fig. 2C), suggesting that in vivo proteolysis in E. coli is not a major issue. The bands with larger molecular weight, particularly the band at the top of the resolving gel (Fig. 2C), are more prominent in case of C147F mutant, suggesting that it aggregates more than WT human arrestin-1.

As the key function of arrestin is specific binding to the active phosphorylated rhodopsin,1,12 we expressed WT and mutant human arrestin-1 with several substitutions in position 147 in cell-free translation in the presence of radioactive amino acids and determined the binding of synthesized radiolabeled proteins to purified P-Rh* in our standard in vitro binding assay.12 Since rhodopsin used in this assay was purified from cow eyes, we used bovine arrestin-1 as a positive control (Fig. 3). We confirmed that the bovine rhodopsin binds the human arrestin-1 essentially as well as the bovine arrestin-1. We previously found this to be the case for the mouse arrestin-1,24,25 which reflects high conservation of both rhodopsin43 and arrestin-114,45 in mammals. Although the human arrestin-1 C147F mutant demonstrated the lowest P-Rh* binding in the group (Fig. 3), it differed from WT by less than 20%. As mouse retinas expressing only 12% and even 4% of WT level of arrestin-1 were perfectly healthy,14 this modest reduction in binding is unlikely to cause photoreceptor health problems in vivo. Moreover, in vitro direct binding assay was performed at 1 nM arrestin-1, whereas the concentration of arrestin-1 in rods is six orders of magnitude higher, exceeding 2 mM.14,46 Another critical functional characteristic of arrestin-1 is high selectivity for the phosphorylated receptor is unlikely to cause severe retinal degeneration. First, several mammalian species with healthy retinas express the phosphorylation-independent arrestin-1 splice variant p44 that demonstrates significantly higher binding to Rh* than does full-length protein.48,49
Second, we previously did not detect retinal degeneration or visual impairment in mice transgenically expressing engineered phosphorylation-independent arrestin-1 mutants, at least at low to physiological levels. The dominant nature of the C147F mutant in humans is also incompatible with the damage being done by its reduced selectivity because the second allele in patients encodes WT arrestin-1. In mice coexpression of WT arrestin-1 partially protects against the damage by the enhanced mutant expressed even at high levels and fully protects against lower levels of the phosphorylation-independent mutant.

However, our structure-function studies suggest that increased Rh* binding is often associated with reduced thermal stability of the protein. Therefore, next we tested the functional integrity of WT and mutant human arrestin-1 forms at elevated temperature.

expression of WT human arrestin-1 was 15.9 ± 0.9 fmol/μL; after high-speed centrifugation, 13.6 ± 0.8 fmol/μL remained in the supernatant). The absolute values (before normalization) were analyzed by 1-way ANOVA with protein as a factor, using Bonferroni post hoc test with correction for multiple comparisons. *P < 0.05, **P < 0.01, as compared to WT human arrestin-1.
The C147F mutant, but not WT human arrestin-1, induces UPR in 661W cells. (A) Representative Western blots for indicated proteins are shown. For BIP and arrestin, 10 μg protein per lane was loaded; for GAPDH, the blots were stripped and reprobed with mouse anti-GAPDH antibody. (B) Quantification of BIP expression in control cells (CO) cells, cells treated with known inducers of UPR 1 μM of thapsigargin (tg1) or 100 μM tunicamycin (tm100), as well as cells transfected with empty vector (pcDNA3) or pcDNA3 encoding the WT human arrestin-1 (DNA per well of six-well plate: 0.56 μg, WT1; 0.64 μg, WT2) or C147F mutant (DNA per well of six-well plate: 0.64 μg, CF1; 0.75 μg, CF2). The data are presented in arbitrary units. The intensity of the bands was normalized to the sum of intensities of all eight bands on the blot in each experiment (n = 5) and multiplied by 10. The data were analyzed by 1-way ANOVA (F(7,16) = 49.4, P < 0.0001), followed by Bonferroni’s post hoc comparisons to corresponding controls (CO or pcDNA3). Statistical significance of the differences is shown, as follows: **P < 0.001, ***P < 0.0001, CO compared to WT1/2, $P < 0.05$ to tm100.

suggest that thermal stability of the C147F mutant is severely compromised, as compared to the WT human arrestin-1. However, physiological human temperature is slightly lower than 37°C. Therefore, we compared functional survival of the WT human arrestin-1 and C147F mutant at this temperature (Fig. 4C) and found that WT retains full functionality for at least 24 hours, whereas the ability of the C147F mutant to bind P-Rh* is significantly reduced after 8 hours and completely gone after 24 hours (Fig. 4C). Thus, the C147F mutant denatures much faster than the WT protein at physiological temperature. Accelerated denaturing appears to be the most likely reason for the photoreceptor loss caused by this mutation in humans15 as process, indicating that the proteasome system and cause cell death via UPR.55 It is interesting that this loss of functional integrity is clearly related to the size of the residue in position 147, as substitution of Cys-147 with relatively bulky Leu or Ile also cause progressive reduction of P-Rh* binding at 42°C, although the survival of these mutants at 1 hour was 2- to 3-fold higher than that of the C147F mutant (Fig. 4A).

Accelerated loss of native folding is expected to induce UPR in expressing cells. Therefore, we tested whether this is the case using photoreceptor-derived cultured 661W cells29 expressing the WT human arrestin-1 or its C147F mutant (Fig. 5). As a positive control, we used 661W cells treated with 1 μM thapsigargin or 100 μM tunicamycin for 1 hour, the...
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**Conclusions**

Our characterization of the WT and mutant human arrestin-1 indicates that reduced protein stability underlies the pathogenesis and dominant nature of disease-causing C147F mutation in arrestin-1. Reduced stability was determined directly as accelerated loss of activity upon incubation at elevated temperature (Fig. 4), induction of UPR in cell culture (Fig. 5), and as indirectly, as reflected in increased binding to a nonpreferred form of rhodopsin, light-activated unphosphorylated Rh* (Fig. 3). However, considering that many species express arrestin-1 splice variant p44, which binds Rh* much better than full-length protein without any adverse consequences, this reduction in selectivity is unlikely to be the underlying cause of photoreceptor death. Collectively, our data suggest that the most likely cause of photoreceptor death in humans carrying C147F mutation in arrestin-1 is its accelerated loss of native folding, resulting in UPR, which causes cell stress and, ultimately, photoreceptor death in patients.

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**References**


