Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 349 (2006) 345–352
BBRC
www.elsevier.com/locate/ybbrc

Ca^{2+}/recoverin dependent regulation of phosphorylation of the rhodopsin mutant R135L associated with retinitis pigmentosa ☆

Ivan I. Senin [b], Laia Bosch [a], Eva Ramon [a,1], Evgeni Yu. Zernii [b], Joan Manyosa [c], Pavel P. Philippov [b], Pere Garriga [a,*]

[a] *Departament d'Enginyeria Química, Universitat Politècnica de Catalunya, 08222 Terrassa, Catalonia, Spain*
[b] *Department of Cell Signalling, A.N. Belozersky Institute of Physico-Chemical Biology, M.V. Lomonosov Moscow State University, 119992 Moscow, Russia*
[c] *Departament de Bioquímica i de Biologia Molecular, Universitat Autònoma de Barcelona, 08193 Bellaterra, Catalonia, Spain*

Received 4 August 2006
Available online 18 August 2006

Abstract

No single molecular mechanism accounts for the effect of mutations in rhodopsin associated with retinitis pigmentosa. Here we report on the specific effect of a Ca^{2+}/recoverin upon phosphorylation of the autosomal dominant retinitis pigmentosa R135L rhodopsin mutant. This mutant shows specific features like impaired G-protein signaling but enhanced phosphorylation in the shut-off process. We now report that R135L hyperphosphorylation by rhodopsin kinase is less efficiently inhibited by Ca^{2+}/recoverin than wild-type rhodopsin. This suggests an involvement of Ca^{2+}/recoverin into the molecular pathogenic effect of the mutation in retinitis pigmentosa which is the cause of rod photoreceptor cell degeneration. This new proposed role of Ca^{2+}/recoverin may be one of the specific features of the proposed new Type III class or rhodopsin mutations associated with retinitis pigmentosa.

Keywords: Rhodopsin phosphorylation; Recoverin; Calcium; Rhodopsin mutation; Retinitis pigmentosa; Rhodopsin kinase; Transducin activation; Visual phototransduction

Retinitis pigmentosa (RP) is a group of retinal degenerative diseases caused by mutations in a number of genes [1], thereby mutations in the visual photoreceptor rhodopsin are the main cause of the autosomal dominant form of the disease (autosomal dominant RP, ADRP) [2]. Over 150 rhodopsin mutations and deletions have been found associated with RP since the first opsin-linked mutation, P23H, was reported [3]. The large number of disease-causing mutations and their spreading throughout the overall protein structure points to the critical importance of a very sophisticated three-dimensional structure for functioning of the visual receptor, and highlights its evolutionary optimal design that plays a key role in the complex phenomenon of vision.

Two types of classification of rhodopsin mutations that cause RP have been proposed. One of them classifies these mutations in two groups depending on the subcellular localization of the mutated protein in the photoreceptor cells and its phenotypic characteristics compared to those of wild-type (WT) rhodopsin [4]. The other classification is based upon several structural and biochemical features of the mutant proteins like chromophore regeneration by means of 11-*cis*-retinal binding, transducin activation rates, and the glycosylation pattern [5]. This classification system was used in the study of a number of RP mutations in rhodopsin, with an added Class III that would correspond to those mutants that showed an intermediate in vitro molecular phenotype (partial chromophore regeneration and

☆ *Abbreviations:* RP, retinitis pigmentosa; WT, wild-type; DM, dodecyl maltoside; G_t, transducin.

* Corresponding author. Fax: +34 937398225.
E-mail address: pere.garriga@upc.edu (P. Garriga).
[1] Present address: Center for Membrane Biology, Department of Biochemistry and Molecular Biology, University of Texas Health Science Center, Houston, TX 77030, USA.

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.08.048

mixed subcellular localization between the ER and the plasma membrane) [6]. There is also a clinical classification of RP that has some analogies with the other classifications [7].

A large number of rhodopsin mutants have been expressed and characterized, and some of them are associated with retinal disease. In spite of this, many RP rhodopsin mutants have not been extensively characterized. We have previously studied some of these mutants, particularly those associated with ADRP [8,9] and congenital night blindness [10]. These mutant genes have been transfected in eukaryotic cells (COS-1) [11] and immunopurified with specific antibodies, like Rho-1D4 which recognizes the C-terminal region of rhodopsin [12].

It is undoubtedly of interest to obtain detailed knowledge of the phenotype of the mutant proteins and to correlate the molecular defects observed with the clinical phenotype of the affected RP patients. This clinical correlation with the laboratory results has turned out to be not straightforward due to aforementioned extreme clinical and genetic heterogeneity of the disease [7]. The onset and the progression of the disease have been found to be very different in relatives carrying the same rhodopsin mutation. Furthermore, there are some RP mutations that apparently do not alter significantly the structure or function of the protein, so it is not clear which might be the cause of the disease in these cases [9]. A comprehensive record of the clinical data for RP patients is also lacking. In spite of these problems, it is necessary to establish a correlation between protein structural features and the RP patient clinical phenotype taking into account the different current hypothesis of how these two aspects may be linked. A theoretical approach based on the rhodopsin sequence data proposes the usefulness of molecular evolutionary analyses for understanding patterns of clinical mutations [13]. Modifications in the native conformation of rhodopsin as a result of RP mutations can result in its altered function, e.g., in alterations of the ability of rhodopsin to activate transducin. In the past decade, several research groups have developed sustained efforts to elucidate the molecular mechanism(s) underlying the retinal RP pathology. The ultimate goal of the research is to contribute to the development of therapeutic solutions to the disease [14].

Protein misfolding has been proposed to be one of the main biochemical causes of RP in the case of some rhodopsin mutations [8,15–17], and molecular chaperones have been also involved in RP. Suppression of WT rhodopsin maturation by mutations linked to ADRP has also been proposed to play a role in the pathophysiology of retinal degeneration [18]. Altered stability and/or function of rhodopsin may also have relevance for a number of given mutations. In this regard, abnormal functioning of rhodopsin resulting in reduced activity or hyperactivity, with regard to G_t activation, could also play a role in RP by altering the stoichiometric balance of the different proteins involved in the phototransduction biochemical reactions. Thus, altered functionality (with increased transducin activation efficiency by rhodopsin) has been proposed as a cause of imbalance that may underlay the deleterious effect observed in these cases [8]. It is important to note that hyperactivity of rhodopsin, in the case of RP, is not necessarily related to constitutive activity of the visual photoreceptor (that is activity in the dark and/or in the absence of 11-*cis*-retinal), but to increased initial activation rate in some rhodopsin mutants. Recently, a gain of function mechanism has also been linked to RP [19]. Some mutations, like those in the C-terminus of rhodopsin, result in mislocalized rhodopsin that has been proposed to damage the cell upon light activation [20]. It has been recently shown that activation of mislocalized rhodopsin is not required to cause retinal degeneration in *Xenopus laevis* [21]. The role of constant activation of the phototransduction pathway on retinal degeneration has also been recently discussed [22]. In this regard, Ca^{2+} lowering and trafficking alterations of visual proteins within the photoreceptor cell may result in morphological changes in the rod outer segments that can lead to photoreceptor cell degeneration [22].

A site of one of the RP mutations is Arg135 at the cytoplasmic side of transmembrane helix III of rhodopsin [23]. This is the site of three ADRP mutations (R135L, R135G, and R135W) [24,25]. Arg135 belongs to the E(D)RY triplet highly conserved throughout the G-protein-coupled receptors superfamily, and is known to be very important for rhodopsin proper functioning. In particular, Arg135 has been proposed to be very important both in the activation of the phototransduction cascade (transducin activation) and also in the deactivation mechanism (phosphorylation by rhodopsin kinase and subsequent arrestin binding) [26]. Arg135 participates in a so-called ionic lock, a set of molecular interactions of an electrostatic nature with adjacent Glu134, (with which it forms an ionic bridge), and amino acids at helix 6 (Glu247 and Thr251). Breakage of these interactions has been proposed as a main requirement for the formation of the active form of the receptor (Ramon et al., to be submitted). RP mutations involving Arg135 are associated with a severe form of the disease [27–29]. A specific clinical phenotype has been documented for a family carrying the R135L mutation, that turns out to be more severe than P23H at the intradiscal side and mutations at the cytoplasmic Pro347 [30].

We have expressed and immunopurified the R135L rhodopsin mutant and analyzed the effect of the mutation on Ca^{2+}/recoverin-sensitive control of rhodopsin phosphorylation. We have found that in the case of the R135L mutant, higher recoverin concentration is needed to cause the same inhibitory effect upon rhodopsin phosphorylation than with WT rhodopsin. This result adds to the current picture of the molecular mechanisms of RP rhodopsin mutations by involving Ca^{2+}/recoverin fine-tuning of rhodopsin shut-off signaling. Thus, the involvement of Ca^{2+}/recoverin modulation of rhodopsin inactivation is a newly proposed mechanism that can play a role in retinal degeneration caused by the R135L mutation, in addition to a

recent study where the specific mechanism to explain photoreceptor cell death caused by this mutation was proposed to be impaired endocytic activity associated with processing of the mutated protein [31]. So far no direct involvement of recoverin in the development of RP has been found [32–34], although it is known that recoverin is associated with another type of retina degeneration, the cancer-associated retinopathy (CAR) [35].

Materials and methods

Purification of rhodopsin from bovine ROS

ROS membranes, prepared by an ultracentrifugation method, were solubilized in buffer B (1.8 mM KH_2PO_4, 10 mM $NaHPO_4$, 137 mM NaCl, and 2.7 mM KCl, pH 7.2, containing 1% dodecyl maltoside (DM)) and immunopurified using a Sepharose column with the covalently attached Rho-1D4 monoclonal antibody against the last amino acids of the C-terminus of rhodopsin (TETSQVAPA). Rhodopsin bound to the antibody column was washed with buffer A (1.8 mM KH_2PO_4, 10 mM $NaHPO_4$, 137 mM NaCl, and 2.7 mM KCl, pH 7.2) and eluted with buffer C (2 mM Na_2HPO_4, pH 6, containing 0.05% DM) containing 100 μM of a 9-mer corresponding to the last nine amino acids of the C-terminus of rhodopsin.

Purification of transducin, rhodopsin kinase, and recoverin

Myristoylated recoverin was heterologously expressed in *Escherichia coli* and purified from a cell extract as described previously [36,37]. Rhodopsin kinase was extracted and purified from bovine ROS employing a modified method of Pulvermüller et al. [38] and described in detail by us previously [39]. Ten to 12 U/ml of rhodopsin kinase extract was typically generated, with the rhodopsin kinase activity expressed as nanomole of ^{32}P incorporated into receptor/min. Purified transducin was prepared as described [40].

Transfection of COS-1 cells and purification of the expressed recombinant rhodopsin

COS-1 cell plates at 80% confluence were transfected with the WT opsin gene by means of a DEAE-dextran method as previously described [11]. Briefly, COS-1 cells in 150-mm diameter tissue culture plates were washed with PBS and incubated in 10 ml transfection cocktail (DEAE dextrane solution containing 20 μg of DNA per plate) for 6 h at 37 °C, 5% CO_2, and saturated humidity. The medium was then aspirated, a solution of chloroquine was added and, the cells were incubated for 3 h at 37 °C, 5% CO_2, and saturated humidity. The medium was then aspirated, a solution of chloroquine was added and, the cells were incubated for 3 h at 37 °C, 5% CO_2, and saturated humidity. The plates were subsequently washed with PBS and incubated for 60 h in 15 ml DMEM +++ per plate at 37 °C, 5% CO_2, and saturated humidity to allow expression of the recombinant protein. Ca. 60 h after the start of the transfection, cells were washed with PBS and collected by scraping the plates with 5 ml of buffer A and centrifuged at 3000 rpm for 5 min at 4 °C. Cell pellets were resuspended in 2 ml of buffer A per tissue culture plate, containing 20 μM of 11-*cis*-retinal and incubated for 4 h at 4 °C. It was necessary to provide exogenous retinal because the mammalian monkey kidney COS-1 cells used for recombinant opsin expression did not endogenously have this molecule. Regenerated samples were solubilized by using a detergent solution, containing final 1% DM and 0.1 mM PMSF, with mild end-to-end continuous stirring for another 1 h under the same conditions. Samples were then centrifuged at 35,000 rpm for 35 min at 4 °C. The supernatant was collected in 15 ml tubes that contained 150 μl of Rho 1D4-coupled Sepharose (see above) and incubated for 3 h at 4 °C for opsin binding. Samples were centrifuged at 3000 rpm and 4 °C for 3 min and were subsequently washed twice with buffer B and buffer C, respectively. Samples were eluted and incubated for 30 min at 4 °C in 500 ml of buffer C containing 100 μM 9-mer peptide. Finally samples were clarified by centrifugation before spectroscopic measurements were carried out.

Transducin assay

The transducin (G_t) activation levels were determined by incorporation of radioactive GTPγ^{35}S in G_t molecules induced by photoactivated WT rhodopsin or rhodopsin mutants essentially as previously described [15]. Briefly, the reaction mixture contained 1 μM transducin, 20 nM rhodopsin, 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM DTT, 0.012% dodecyl maltoside, and 3 μM GTPγ^{35}S. The reaction was started by illumination of the sample for 30 s and stopped 1 h later by the addition of a "stop solution", containing 10 mM Tris–HCl, pH 7.4, 100 mM NaCl, and 10 mM EDTA. The unbound GTPγ^{35}S was removed by microfiltration. The amount of GTPγ^{35}S bound to G_t was determined by Cherenkov counting.

Phosphorylation assay

ROS and COS membranes. Phosphorylation of rhodopsin in ROS or COS membranes (see legend to Fig. 1) was carried out in the reaction mixture (250 μl) containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 3 mM $MgCl_2$, 200 μM [γ-^{32}P]ATP ($1 - 3 \times 10^5$ cpm/nmol), and 0.1 U of rhodopsin kinase. Immediately after illumination of the mixture (100% bleaching of rhodopsin), [γ-^{32}P]ATP was added to start the reaction, which was stopped 1–40 min later by the addition of 100 μl buffer containing 50 mM ATP and 20 mM EDTA. The mixture was mixed with Rho-1D4 antibody immobilized on protein A Sepharose, and rhodopsin was eluted with 2× the SDS–PAGE sample buffer. After SDS–PAGE of the samples, the bands corresponding to rhodopsin were cut out, and ^{32}P incorporation was determined by Cherenkov counting.

"Soluble" system. Phosphorylation of purified rhodopsin from solubilized membranes was carried out in the reaction mixture (30 μl) containing 20 μM Tris–HCl (pH 7.5), 150 μM NaCl, 3 μM $MgCl_2$, 200 μM [γ-^{32}P]ATP ($1 - 3 \times 10^5$ cpm/nmol), 0.1 U of rhodopsin kinase, and 0.02% DM, rhodopsin or rhodopsin mutant with or without recoverin as indicated (see legends to Figs. 3 and 4). Immediately after illumination of the mixture (100% bleaching of rhodopsin), [γ-^{32}P]ATP was added to start the reaction, which was stopped 1–40 min later by the addition of 2× SDS–PAGE sample buffer. After SDS–PAGE of the samples, the bands corresponding to rhodopsin were cut out, and ^{32}P incorporation was determined by Cherenkov counting.

Results and discussion

UV–vis absorbance spectra of purified recombinant WT rhodopsin and of the R135L mutant are presented in Fig. 1A. The spectrum for the mutant is very similar to that previously reported [41]. No chromophore regeneration was detected from photobleaching difference spectra of regenerated 293 cell membranes [4]. The chromophore yield—as judged by absorption at 280 nm—seems similar for the two proteins, and the chromophoric visible band is located at 500 nm in both cases. However, careful inspection of the spectra reveals a lower chromophore formation for the R135L RP mutant when compared to WT rhodopsin: the A_{280}/A_{500} ratio was equal to 2.5 and 1.8 in the case of the mutant and WT rhodopsin, respectively. This difference can be due to: (i) a lower ability of the mutant opsin to bind 11-*cis*-retinal, suggesting some degree of structural misfolding, (ii) impaired accessibility to the binding pocket for the retinal, or (iii) some degree of instability of the

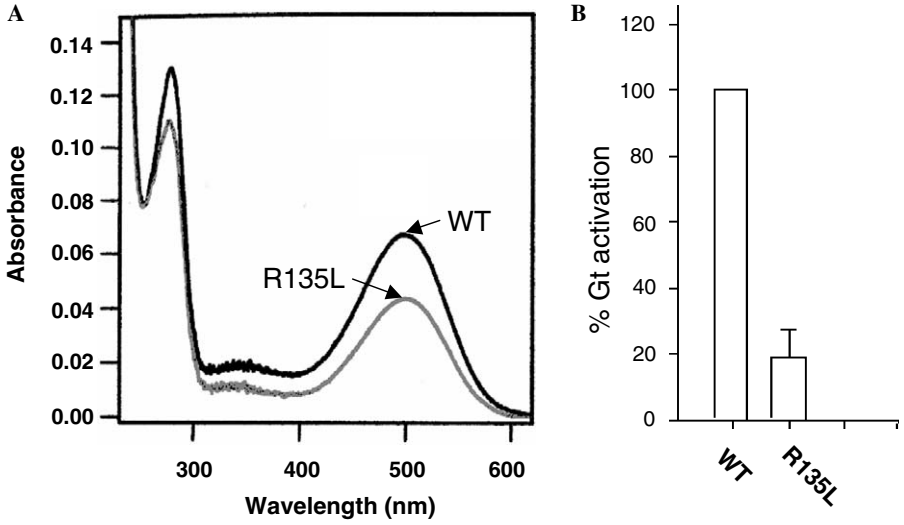

Fig. 1. (A) UV–visible absorption spectra of WT rhodopsin and the rhodopsin mutant R135L in 2 mM sodium phosphate, pH 6.0, 0.05% DM. (B) G_t activation of WT rhodopsin (WT) and the R135L mutant (R135L) after illumination with light of $\lambda > 495$ nm.

formed chromophore that would be lost during the purification procedure.

Both two latter hypotheses seem to be more plausible than misfolding of the receptor as the mutation is located at the cytoplasmic boundary of the rhodopsin helix 3, i.e. in a region very distant to the 11-*cis*-retinal binding pocket. This makes unlikely that the increased A_{280}/A_{500} ratio reflects structural misfolding of the receptor. We have previously reported that the dark state of the purified R135L mutant is somehow more thermally unstable than WT rhodopsin [8]. In addition, the hydroxylamine reactivity of this mutant in the dark is slightly modified with regard to the WT behavior [8]. The electrophoretic mobility of the mutant was found to be similar to the WT, indicating that the mutant was normally glycosylated and processed in the COS-1 cells to the plasma membrane and not retained in any other subcellular compartment. These differences argue against protein misfolding and suggest subtle differences in the dark conformation of the mutant.

The functionality of the mutant rhodopsin was also determined by means of a radioactive binding assay. It was found that the activation of G_t was severely impaired giving only 20% of the total activation when compared to WT rhodopsin under the same experimental conditions (Fig. 1B). Previous studies found no activation at all for this mutant [41] and about 50% of the initial activation rate in a more recent study where the activation was measured by fluorescence spectroscopy [8].

Several aspects can be taken into account to reconcile the apparent discrepancy in the results obtained with the same kind of sample, i.e. detergent purified mutant protein. It should be noted that the rhodopsin and G_t concentrations used in the three studies are different. More rhodopsin is used in our previous [8] and current studies than that in the study where no activity was detected [41]. If the affinity of rhodopsin for G_t is lowered, then increasing concentrations of rhodopsin in the assay can lead to detecting some activity that otherwise will not be seen. In the case of the R135L mutant our previous result showed a decrease of about 50% in the initial rate of activation [8] but the total activation was lower. This could also be explained by a faster decay of the active conformation metarhodopsin II in spite of the higher rhodopsin concentration used in this study. Overall, the results of our investigation of G_t activation by the R135L rhodopsin mutant, indicate that this mutant has impaired ability to activate the phototransduction cascade. A possible explanation that can be suggested is that R135 in WT rhodopsin is actually involved in the physical binding to G_t. Thus, mutation at this site will be perturbing normal rhodopsin-G_t binding in the corresponding molecular recognition event.

Although the activation of G_t is severely affected as a result of the rhodopsin mutation, the contrary is true for the inactivation through phosphorylation by rhodopsin kinase. Fig. 2A shows results of phosphorylation of recombinant WT rhodopsin in COS membranes and retinal rhodopsin in ROS membranes. The time dependence of rhodopsin phosphorylation, in ROS and COS membranes, was found to be very similar (data not shown). However, our data highlight differences in the level of phosphorylation of these two forms of rhodopsin. In all our experiments, phosphorylation reactions of recombinant WT rhodopsin in COS cell membranes had rates between 57% and 72% of those with retinal rhodopsin in ROS membranes. The observed difference between rhodopsin phosphorylation in ROS and COS-1 cell membranes is likely due to differences in phospholipid composition, the importance of which for rhodopsin structure and function has been stressed in the last years [42,43]. Particularly relevant is the role of retinal specific lipids, like docosohexanoic acid, in rhodopsin structure, function, and activation [44,45].

In order to evaluate whether the substrate properties of recombinant rhodopsin in the phosphorylation reaction

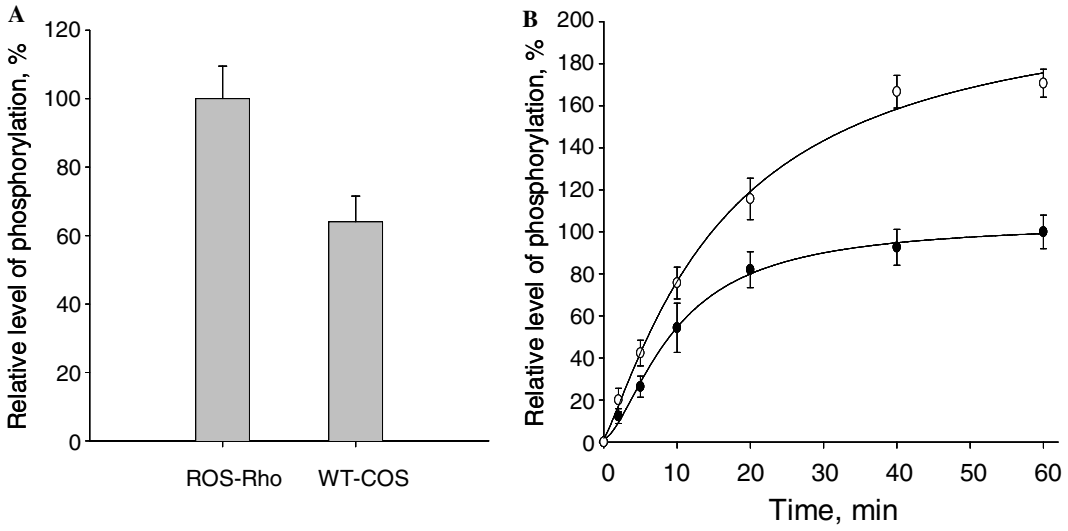

Fig. 2. (A) Phosphorylation of rhodopsin in the content of ROS (ROS-Rho) and COS-1 cell (WT-COS) membranes. A reconstituted system containing urea-washed ROS or COS membranes (1 μg of rhodopsin), rhodopsin kinase, and [γ-^{32}P]ATP was incubated for 40 min and processed as described in Materials and methods. Each data point represents the mean ± SEM for five experiments. (B) Time dependence of phosphorylation of purified retinal ROS rhodopsin (●) and the R135L mutant (○) in a soluble system. Employing the standard rhodopsin-phosphorylation assay in a soluble system (see Materials and methods), the time course for ^{32}P incorporation was followed over 60 min for incubation mixtures containing 0.1 μM rhodopsin purified from ROS (●) or COS (○) membranes.

catalyzed by rhodopsin kinase are similar to those of retinal rhodopsin, we compared the properties of both forms of rhodopsin in a soluble system consisting of purified proteins. For experiments in the soluble system, recombinant WT and retinal rhodopsin were purified by using the specific Rho-1D4 monoclonal antibody and ConA column, respectively. The behavior of recombinant WT and retinal rhodopsin in terms of their phosphorylation was very similar. The corresponding kinetic parameters for recombinant WT and retinal rhodopsin were the following: K_m 0.54 and 0.51 μM, respectively, and V_{max} 34 and 37 pmol/min/μg of rhodopsin kinase, respectively (data not shown). These results strengthen the mentioned idea that the observed differences in phosphorylation level of rhodopsin in ROS and COS membranes are due to differences in the phospholipid matrix composition of these membranes.

It was previously reported that some rhodopsin mutants at position 135, in particular the R35L mutant, displayed a higher level of light-induced phosphorylation than that of WT rhodopsin, and an increase in their affinity for visual arrestin [26]. Therefore, we hypothesized that these changes in affinity of the R135L rhodopsin mutant for rhodopsin kinase might lead to changes in the Ca^{2+}/recoverin dependent control of rhodopsin phosphorylation. In order to verify this hypothesis, we investigated the Ca^{2+}-dependent effect of recoverin upon phosphorylation, of retinal rhodopsin and the R135L mutant, catalyzed by rhodopsin kinase.

We found that the R135L mutant was 35–70% more phosphorylated by rhodopsin kinase in comparison with recombinant WT rhodopsin (Fig. 2B). In the absence of recoverin, phosphorylation of WT rhodopsin and the R135L mutant was Ca^{2+}-insensitive (data not shown). In the presence of saturating Ca^{2+} concentrations and 2 μM of recoverin, the level of WT rhodopsin phosphorylation was decreased by 40–50%. However, phosphorylation of the R135L mutant was less sensitive to Ca^{2+}/recoverin: in this case, the phosphorylation level was decreased only by 20–25% (data not shown). The rhodopsin kinase activity dependence on rhodopsin concentration in purified detergent solubilized samples was investigated (Fig. 3). A higher level of phosphorylation is detected in the case of the mutant in agreement with results presented in Fig. 2B. Fur-

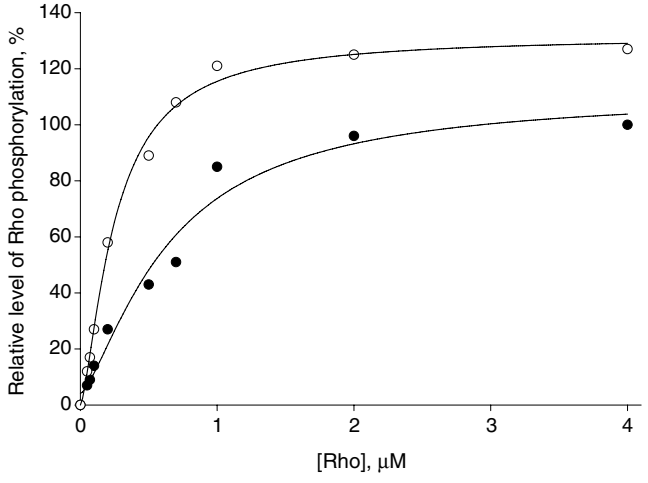

Fig. 3. Concentration dependence of WT rhodopsin (●) and the R135L mutant (○) phosphorylation in a soluble system. A reconstituted system containing purified retinal ROS rhodopsin or R135L mutant (0.1–4 μM of rhodopsin), rhodopsin kinase, and [γ-^{32}P]ATP was incubated for 40 min and processed as described in Materials and methods. Each data point represents the mean ± SEM for two experiments.

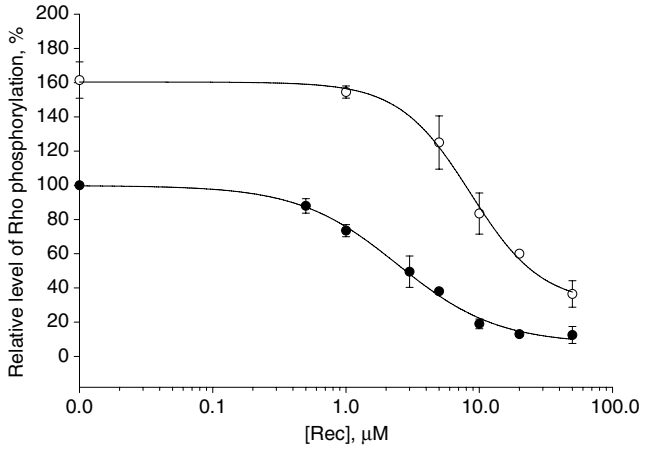

Fig. 4. Effect of recoverin on phosphorylation of retinal ROS rhodopsin (●) and the R135L mutant (○) in a soluble system. Known concentrations of recoverin (0.1–50 μM) were added to the soluble rhodopsin-phosphorylation assay system (see Materials and methods) containing 0.1 μM rhodopsin. The level of ^{32}P incorporation was determined. Each data point represents the mean ± SEM for three separate experiments. K_{50} for WT rhodopsin and the R135L mutant is equal to 2.2 and 9 μM, respectively.

thermore, a faster kinetics of phosphorylation is also seen for the R135L mutant when compared with WT. The dependence of the recoverin inhibitory effect upon phosphorylation of retinal rhodopsin and the mutant R135L on recoverin concentration is shown in Fig. 4. It should be noted that the inhibitory action of recoverin on phosphorylation of WT rhodopsin is observed at a significantly lower concentration of recoverin than in the case of the R135L mutant. EC_{50} value is 2.2 μM for WT rhodopsin phosphorylation, whereas it is increased to 9 μM in the case of the mutant.

Recoverin is a small myristoylated calcium binding protein [46] that binds to rhodopsin kinase in the presence of high Ca^{2+} concentration acting as an inhibitor of the kinase activity [47]. Recent studies have provided information about the structural domains of this calcium-sensor important for its interaction with rhodopsin kinase [48,49]. These data provide support for a model in which recoverin prevents rhodopsin phosphorylation by sterically blocking a region of rhodopsin kinase essential for its interaction with rhodopsin [49]. Thus, previous results suggest an important role for recoverin in the deactivation pathway of visual phototransduction. In our present study, we propose an involvement of Ca^{2+}/recoverin regulation in the desensitization process of the ADRP rhodopsin mutant R135L. Previous works studied the possible involvement of recoverin in the molecular pathophysiology of RP, but no direct link was found between recoverin and RP [32–34]. On the other side, recoverin has been found linked to cancer-associated retinopathy [35], and this broadens the physiological roles in which this Ca^{2+}-binding protein is proposed to be involved.

Hyperphosphorylation and high-affinity binding to arrestin for R135L has been shown to result in an impairment of the endocytic activity and this has been proposed to be the cause of the pathological effect associated with this specific mutation [31]. This has led to the proposal that 135 mutations would belong to a new Class III of RP mutants characterized for a clearly distinct phenotype [31].

Our results suggest an involvement for Ca^{2+}/recoverin in the regulation of rhodopsin phosphorylation by rhodopsin kinase in the case of the R135L mutant. This effect could take place for molecules of the apoprotein that can be constitutively phosphorylated by rhodopsin kinase and have bound arrestin, but also in the case of the majority of rhodopsin molecules that can be regenerated and therefore are sensitive to light. Assuming that these molecules reach the plasma membrane and follow a classical photoactivation pathway, this mechanism of fine regulation by means of Ca^{2+}/recoverin can also come in play for receptor desensitization. Little or no G_t activation would result in only slight decrease in intracellular Ca^{2+} in photoreceptor cells (compared to the normal physiological response, i.e. important decrease in intracellular Ca^{2+}). Thus, Ca^{2+} would be more readily available for recoverin, and more recoverin will be needed to produce the same inhibitory effect in the case of the R135L mutant than in the case of WT rhodopsin. In summary, our current results—obtained in a controlled experimental system with purified proteins—show that an effect at the recoverin level can be associated with an RP mutation, and that this effect must be specifically due to the mutation because no modification of the kinase or recoverin has been made. This is the first time that alterations at the recoverin level, in the deactivation of the phototransduction cascade, are linked to RP.

Acknowledgments

This work was supported by grants from Ministerio de Educación y Ciencia (SAF2005-08148-C02-04 to P.G.), NATO Scientific Programme, INTAS (03-51-4548) to I.I.S., J.M., and P.G., FEBS Fellowship program (to E.Yu.Z.), and Russian Foundation for Basic Research to P.P.Ph. (06-04-48018, 04-04-04001) and I.I.S. (03-04-48909, 06-04-48761).

References

[1] C. Rivolta, D. Sharon, M.M. DeAngelis, T.P. Dryja, Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns, Hum. Mol. Genet. 11 (2002) 1219–1227.
[2] A. Kennan, A. Aherne, P. Humphries, Light in retinitis pigmentosa, Trends Genet. 21 (2005) 103–110.
[3] G.J. Farrar, P. McWilliam, D.G. Bradley, P. Kenna, M. Lawler, E.M. Sharp, M.M. Humphries, H. Eiberg, P.M. Conneally, J.A. Trofatter, et al., Autosomal dominant retinitis pigmentosa: linkage to rhodopsin and evidence for genetic heterogeneity, Genomics 8 (1990) 35–40.
[4] C.H. Sung, B.G. Schneider, N. Agarwal, D.S. Papermaster, J. Nathans, Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa, Proc. Natl. Acad. Sci. USA 88 (1991) 8840–8844.

[5] T. Doi, R.S. Molday, H.G. Khorana, Role of the intradiscal domain in rhodopsin assembly and function, Proc. Natl. Acad. Sci. USA 87 (1990) 4991–4995.
[6] S. Kaushal, H.G. Khorana, Structure and function in rhodopsin. 7. Point mutations associated with autosomal dominant retinitis pigmentosa, Biochemistry 33 (1994) 6121–6128.
[7] E. Ramon, L.J. del Valle, P. Garriga, Molecular biology of retinitis pigmentosa: therapeutic implications, Curr. Pharmacogenomics 2 (2004) 339–349.
[8] A. Andres, P. Garriga, J. Manyosa, Altered functionality in rhodopsin point mutants associated with retinitis pigmentosa, Biochem. Biophys. Res. Commun. 303 (2003) 294–301.
[9] L. Bosch, E. Ramon, L.J. del Valle, P. Garriga, Structural and functional role of helices I and II in rhodopsin. A novel interplay evidenced by mutations at Gly-51 and Gly-89 in the transmembrane domain, J. Biol. Chem. 278 (2003) 20203–20209.
[10] E. Ramon, J.L. del Valle, P. Garriga, Unusual thermal and conformational properties of the rhodopsin congenital night blindness mutant Thr-94 → Ile, J. Biol. Chem. 278 (2003) 6427–6432.
[11] D.D. Oprian, R.S. Molday, R.J. Kaufman, H.G. Khorana, Expression of a synthetic bovine rhodopsin gene in monkey kidney cells, Proc. Natl. Acad. Sci. USA 84 (1987) 8874–8878.
[12] R.S. Molday, D. MacKenzie, Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes, Biochemistry 22 (1983) 653–660.
[13] A.D. Briscoe, C. Gaur, S. Kumar, The spectrum of human rhodopsin disease mutations through the lens of interspecific variation, Gene 332 (2004) 107–118.
[14] H.F. Mendes, J. van der Spuy, J.P. Chapple, M.E. Cheetham, Mechanisms of cell death in rhodopsin retinitis pigmentosa: implications for therapy, Trends Mol. Med. 11 (2005) 177–185.
[15] P. Garriga, X. Liu, H.G. Khorana, Structure and function in rhodopsin: correct folding and misfolding in point mutants at and in proximity to the site of the retinitis pigmentosa mutation Leu-125 → Arg in the transmembrane helix C, Proc. Natl. Acad. Sci. USA 93 (1996) 4560–4564.
[16] X. Liu, P. Garriga, H.G. Khorana, Structure and function in rhodopsin: correct folding and misfolding in two point mutants in the intradiscal domain of rhodopsin identified in retinitis pigmentosa, Proc. Natl. Acad. Sci. USA 93 (1996) 4554–4559.
[17] J. Hwa, P.J. Reeves, J. Klein-Seetharaman, F. Davidson, H.G. Khorana, Structure and function in rhodopsin: further elucidation of the role of the intradiscal cysteines, Cys-110, -185, and -187, in rhodopsin folding and function, Proc. Natl. Acad. Sci. USA 96 (1999) 1932–1935.
[18] R.S. Rajan, R.R. Kopito, Suppression of wild-type rhodopsin maturation by mutants linked to autosomal dominant retinitis pigmentosa, J. Biol. Chem. 280 (2005) 1284–1291.
[19] J. Lem, G.L. Fain, Constitutive opsin signaling: night blindness or retinal degeneration? Trends Mol. Med. 10 (2004) 150–157.
[20] P.D. Alfinito, E. Townes-Anderson, Activation of mislocalized opsin kills rod cells: a novel mechanism for rod cell death in retinal disease, Proc. Natl. Acad. Sci. USA 99 (2002) 5655–5660.
[21] B.M. Tam, G. Xie, D.D. Oprian, O.L. Moritz, Mislocalized rhodopsin does not require activation to cause retinal degeneration and neurite outgrowth in *Xenopus laevis*, J. Neurosci. 26 (2006) 203–209.
[22] G.L. Fain, Why photoreceptors die (and why they don't), Bioessays 28 (2006) 344–354.
[23] K. Palczewski, G protein-coupled receptor rhodopsin, Annu. Rev. Biochem. 75 (2006) 743–767.
[24] C.H. Sung, C.M. Davenport, J.C. Hennessey, I.H. Maumenee, S.G. Jacobson, J.R. Heckenlively, R. Nowakowski, G. Fishman, P. Gouras, J. Nathans, Rhodopsin mutations in autosomal dominant retinitis pigmentosa, Proc. Natl. Acad. Sci. USA 88 (1991) 6481–6485.
[25] J.P. Macke, C.M. Davenport, S.G. Jacobson, J.C. Hennessey, F. Gonzalez-Fernandez, B.P. Conway, J. Heckenlively, R. Palmer, I.H. Maumenee, P. Sieving, et al., Identification of novel rhodopsin mutations responsible for retinitis pigmentosa: implications for the structure and function of rhodopsin, Am. J. Hum. Genet. 53 (1993) 80–89.
[26] W. Shi, C.D. Sports, D. Raman, S. Shirakawa, S. Osawa, E.R. Weiss, Rhodopsin arginine-135 mutants are phosphorylated by rhodopsin kinase and bind arrestin in the absence of 11-*cis*-retinal, Biochemistry 37 (1998) 4869–4874.
[27] V. Ponjavic, M. Abrahamson, S. Andreasson, B. Ehinger, G. Fex, Autosomal dominant retinitis pigmentosa with a rhodopsin mutation (Arg-135-Trp). Disease phenotype in a Swedish family, Acta Ophthalmol. Scand. 75 (1997) 218–223.
[28] M.R. Pannarale, B. Grammatico, A. Iannaccone, R. Forte, C. DeBernardo, L. Flagiello, E.M. Vingolo, G. Del Porto, Autosomal-dominant retinitis pigmentosa associated with an Arg-135-Trp point mutation of the rhodopsin gene. Clinical features and longitudinal observations, Ophthalmology 103 (1996) 1443–1452.
[29] S. Andreasson, B. Ehinger, M. Abrahamson, G. Fex, A six-generation family with autosomal dominant retinitis pigmentosa and a rhodopsin gene mutation (arginine-135-leucine), Ophthalmic. Paediatr. Genet. 13 (1992) 145–153.
[30] K.T. Oh, D.M. Oh, R.G. Weleber, E.M. Stone, A. Parikh, J. White, K.A. Deboer-Shields, L. Streb, C. Vallar, Genotype–phenotype correlation in a family with Arg135Leu rhodopsin retinitis pigmentosa, Br. J. Ophthalmol. 88 (2004) 1533–1537.
[31] J.Z. Chuang, C. Vega, W. Jun, C.H. Sung, Structural and functional impairment of endocytic pathways by retinitis pigmentosa mutant rhodopsin–arrestin complexes, J. Clin. Invest. 114 (2004) 131–140.
[32] J. Greenberg, R. Goliath, P. Beighton, R. Ramesar, A new locus for autosomal dominant retinitis pigmentosa on the short arm of chromosome 17, Hum. Mol. Genet. 3 (1994) 915–918.
[33] M. Bayes, D. Valverde, S. Balcells, D. Grinberg, L. Vilageliu, J. Benitez, C. Ayuso, M. Beneyto, M. Baiget, R. Gonzalez-Duarte, Evidence against involvement of recoverin in autosomal recessive retinitis pigmentosa in 42 Spanish families, Hum. Genet. 96 (1995) 89–94.
[34] A.H. Parminder, A. Murakami, G. Inana, E.L. Berson, T.P. Dryja, Evaluation of the human gene encoding recoverin in patients with retinitis pigmentosa or an allied disease, Invest. Ophthalmol. Vis. Sci. 38 (1997) 704–709.
[35] H. Ohguro, M. Nakazawa, Pathological roles of recoverin in cancer-associated retinopathy, Adv. Exp. Med. Biol. 514 (2002) 109–124.
[36] I.I. Senin, T. Fischer, K.E. Komolov, D.V. Zinchenko, P.P. Philipov, K.W. Koch, Ca^{2+}-myristoyl switch in the neuronal calcium sensor recoverin requires different functions of Ca^{2+}-binding sites, J. Biol. Chem. 277 (2002) 50365–50372.
[37] I.I. Senin, S.A. Vaganova, O.H. Weiergräber, N.S. Ergorov, P.P. Philippov, K.W. Koch, Functional restoration of the Ca^{2+}-myristoyl switch in a recoverin mutant, J. Mol. Biol. 330 (2003) 409–418.
[38] A. Pulvermüller, K. Palczewski, K.P. Hofmann, Interaction between photoactivated rhodopsin and its kinase: stability and kinetics of complex formation, Biochemistry 32 (1993) 14082–14088.
[39] I.I. Senin, K.R. Dean, A.A. Zargarov, M. Akhtar, P.P. Philippov, Recoverin inhibits the phosphorylation of dark-adapted rhodopsin more than it does that of bleached rhodopsin: a possible mechanism through which rhodopsin kinase is prevented from participation in a side reaction, Biochem. J. 321 (1997) 551–555.
[40] H. Kuhn, Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes, Nature 283 (1980) 587–589.
[41] M.C. Min, T.A. Zvyaga, A.M. Cypess, T.P. Sakmar, Characterization of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. Mutations on the cytoplasmic surface affect transducin activation, J. Biol. Chem. 268 (1993) 9400–9404.
[42] A. Grossfield, S.E. Feller, M.C. Pitman, A role for direct interactions in the modulation of rhodopsin by omega-3 polyunsaturated lipids, Proc. Natl. Acad. Sci. USA 103 (2006) 4888–4893.

[43] E. Ramon, J. Marron, L.J. del Valle, L. Bosch, A. Andres, J. Manyosa, P. Garriga, Effect of dodecyl maltoside detergent on rhodopsin stability and function, Vision Res. 43 (2003) 3055–3061.
[44] R.E. Anderson, J.S. Penn, Environmental light and heredity are associated with adaptive changes in retinal DHA levels that affect retinal function, Lipids 39 (2004) 1121–1124.
[45] S.E. Feller, K. Gawrisch, Properties of docosahexaenoic-acid-containing lipids and their influence on the function of rhodopsin, Curr. Opin. Struct. Biol. 15 (2005) 416–422.
[46] A.M. Dizhoor, S. Ray, S. Kumar, G. Niemi, M. Spencer, D. Brolley, K.A. Walsh, P.P. Philipov, J.B. Hurley, L. Stryer, Recoverin: a calcium sensitive activator of retinal rod guanylate cyclase, Science 251 (1991) 915–918.
[47] A. Polans, W. Baehr, K. Palczewski, Turned on by Ca^{2+}! The physiology and pathology of Ca(2+)-binding proteins in the retina, Trends Neurosci. 19 (1996) 547–554.
[48] K.E. Komolov, D.V. Zinchenko, V.A. Churumova, S.A. Vaganova, O.H. Weiergraber, I.I. Senin, P.P. Philippov, K.W. Koch, One of the Ca^{2+}-binding sites of recoverin exclusively controls interaction with rhodopsin kinase, Biol. Chem. 386 (2005) 285–289.
[49] M.K. Higgins, D.D. Oprian, G.F. Schertler, Recoverin binds exclusively to an amphipathic peptide at the N-terminus of rhodopsin kinase, inhibiting rhodopsin phosphorylation without affecting catalytic activity of the kinase, J. Biol. Chem. 281 (2006) 19426–19432.