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Partial Agonist Activity of 11-*cis*-Retinal in Rhodopsin Mutants*

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Rhodopsin, the photoreceptor molecule of the vertebrate rod cell, is a G protein-coupled receptor. Rhodopsin consists of the opsin apoprotein and its 11-*cis*-retinal chromophore, which is covalently bound to a specific lysine residue by a stable protonated Schiff base linkage. Rhodopsin activation occurs when light causes photoisomerization of the 11-*cis* chromophore to its all-*trans* form. The all-*trans* chromophore is the receptor agonist. The 11-*cis*-retinylidene chromophore is analogous pharmacologically to a potent inverse agonist of the receptor. We report here that replacement of a highly conserved glycine residue (Gly^{121}) causes 11-*cis*-retinal to become a pharmacologic partial agonist. Although the mutant apoproteins do not display constitutive activity, they are active in the dark when bound to an 11-*cis*-retinylidene chromophore, or to a "locked" chromophore analogue, Ret-7. The degree of partial agonism is directly related to the size of the amino acid replacement at position 121, and it can be reversed by a specific second-site replacement of Phe^{261}. Thus, mutation of Gly^{121} in rhodopsin causes 11-*cis*-retinal to act as a partial agonist rather than an inverse agonist, allowing the mutant pigment to activate transducin in the dark.

The absolute threshold for visual perception is ensured by the extremely low thermal noise of the visual system, which stems from the low probability of spontaneous activation at each step of the vision signal transduction cascade (1–3). The two main components of the electrical noise of rod cells are a photon-like component originating from thermal activation of rhodopsin (Rho)[1] (4, 5) and a continuous fluctuation in the membrane current arising from the spontaneous activation of cGMP phosphodiesterase (6). Transducin (G_t) activation by Rho containing the covalently bound 11-*cis*-retinal chromophore in the dark does not contribute significantly to thermal noise. The low intrinsic activity of the opsin apoprotein observed at low pH and the G_t signaling by constitutively activate mutants, such as E113Q, can be inhibited by 11-*cis*-retinal (7–9). Therefore, 11-*cis*-retinal can be defined as a potent natural inverse agonist of opsin (10, 11). *In vivo* studies have also shown that 11-*cis*-retinal is capable of re-sensitizing bleached photoreceptors and helps the recovery of bleach adaptation (12, 13). The extremely low, if any, activity of Rho with its bound 11-*cis*-retinal ligand is an essential aspect of the physiology of the vertebrate visual system.

Gly^{121} in transmembrane (TM) helix 3 of Rho is strictly conserved in all of the visual opsins. Replacement of Gly^{121} with residues of increasing size results in a progressive blue shift in the λ_{max} of the mutant pigments and increased G_t activation by mutant opsins in the presence of ATR (14). Here, we report that 11-*cis*-retinal can activate Gly^{121} mutant opsins in the dark. The Gly^{121} mutant opsins do not display significant constitutive activity. However, the addition of 11-*cis*-retinal, or a "locked" retinal analogue, results in significant GTPγS uptake by G_t even in the absence of light. Therefore, we conclude that 11-*cis*-retinal serves as a partial agonist in the Gly^{121} mutants rather than as an inverse agonist as it does in wild-type opsin. The mechanism of partial agonism of 11-*cis*-retinal in the Gly^{121} mutant opsins may be related to steric interactions that affect the relative orientation of TM helices 3 and 6.

EXPERIMENTAL PROCEDURES

The construction of opsin mutants was described previously (14, 15). Each of the mutant pigments was purified in dodecyl maltoside (DM) detergent buffer solution, and pigment concentrations were determined according to absorbance at the visible λ_{max} and the ϵ value reported previously (14, 15). G_t activation assays were carried out as described (14, 16). Dark activity was measured under dim red light. The 11-*cis*-retinal analogue Ret-7 (Fig. 2*A*) was synthesized as described previously (17). The 11-*cis*-retinal was obtained from Dr. R. K. Crouch, and the all-*trans*-retinal (ATR) was purchased from Sigma.

RESULTS

Rho purified in DM does not activate G_t at detectable levels in the absence of light (Fig. 1*A*). In contrast, the G121L mutant regenerated with 11-*cis*-retinal displayed an abnormally high level of G_t activation in the dark (Fig. 1*A*). Upon illumination, mutant pigment G121L and Rho exhibited identical abilities to catalyze GTPγS uptake by G_t (Fig. 1*B*). The purified sample of mutant G121L is a mixture of G121L pigment with a visible λ_{max} value of 475 nm and free G121L opsin as judged by the high spectral ratio (A_{280}/A_{475}) of 8.2 ± 1.1 (14). Since G121L opsin purified in parallel did not activate G_t, the G_t activation shown in Fig. 1*A* originated from G121L pigment rather than from constitutive activity of the G121L opsin. Purified mutant pigment G121I also displayed significant dark activity (not shown). No detectable dark activity was observed for the Gly^{121} mutant pigments G121A, G121S, G121T, or G121V purified in DM. Purified mutant G121W also did not activate G_t, but this mutant failed to form a pigment after the purification procedure (14).

Assays carried out in COS cell membranes showed that Gly^{121} mutants could activate G_t in the presence of ATR and

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[1] The abbreviations used are: Rho, rhodopsin; ATR, all-*trans*-retinal; DM, *n*-dodecyl-β-D-maltoside; G_t, transducin; TM, transmembrane; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

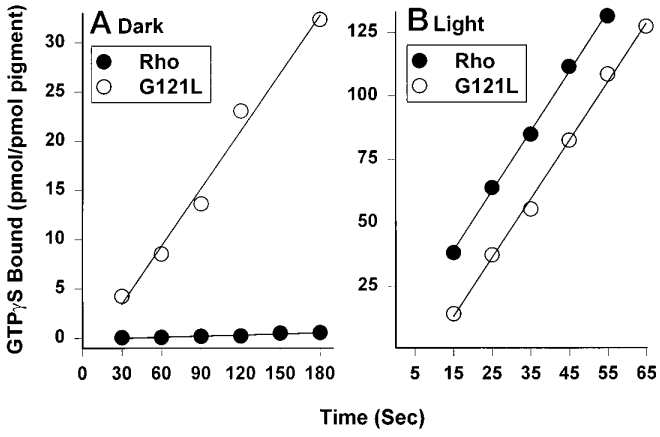

FIG. 1. **Rho mutant G121L catalyzed GTPγS uptake by G_t in the dark.** The mutant pigment G121L was purified after incubation with 11-*cis*-retinal. *A*, pigment G121L displayed significant ability to activate G_t in darkness. Under identical conditions Rho did not activate G_t. *B*, under continuous illumination, mutant pigment G121L and Rho were nearly identical in catalyzing GTPγS uptake by G_t.

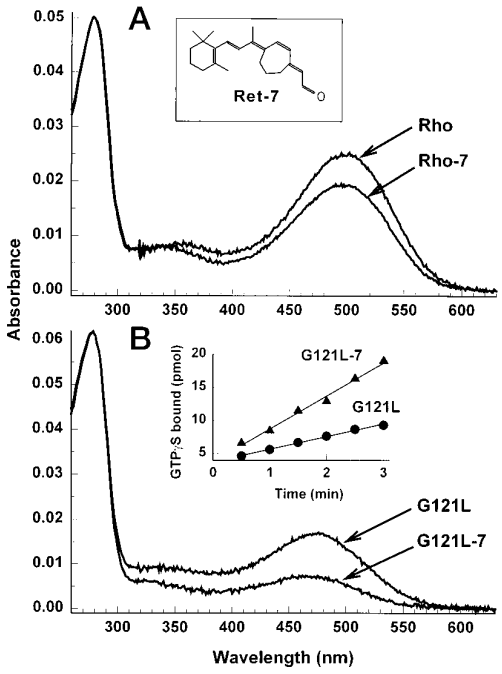

FIG. 2. **Absorption spectroscopy of analogue and mutant pigments.** *A*, UV-visible absorption spectra of Rho and opsin regenerated with chromophore analogue Ret-7 (Rho-7). The structure of the Ret-7 analogue with the $C_{11}=C_{12}$ bond locked in the *cis* conformation by a 7-membered ring is shown in the *inset*. Rho and Rho-7 were purified after incubation with 11-*cis*-retinal and Ret-7, respectively. Absorbance at 280 nm for both pigments is normalized to 0.05. The visible λ_{max} values of Rho and Rho-7 were 500 nm and 498 nm, respectively. *B*, UV-visible absorption spectra of mutant pigment G121L and G121L opsin regenerated with the chromophore analogue Ret-7 (G121L-7). G121L and G121L-7 were purified after incubation with 11-*cis*-retinal and Ret-7, respectively. Absorbance at 280 nm for both pigments is normalized. The visible λ_{max} values of G121L and G121L-7 were 475 nm and 470 nm, respectively. G_t activation in the dark by purified pigments G121L and G121L-7 is shown in the *inset*. Both mutant pigments were capable of activating G_t in darkness.

that G121L incorporated ATR much faster than the 11-*cis* isomer (14). It is therefore conceivable that the apparent light-independent activation of the Gly^{121} mutants with 11-*cis*-retinal described above was due to the presence of ATR, which could originate from: 1) contamination of the 11-*cis*-retinal solution, 2) thermal isomerization of 11-*cis* to ATR during the incubation with opsin, or 3) a higher thermal isomerization rate of 11-*cis* to ATR in the retinal-binding site of the mutant pigment. To control for these possible artifacts, the G121L mutant opsin was incubated with ATR and then subjected to the same purification procedure. Although the opsin yield was normal judging by the 280 nm absorbance, no detectable pigment was observed either at 380 nm or in the visible region. The purified product did not activate G_t. These findings argue against the possibility that the dark activity of the G121L pigment is due to the presence of ATR during ligand incorporation.

The thermal isomerization rate of 11-*cis*-retinal to ATR is extremely low in Rho (4). To rule out increased thermal isomerization from 11-*cis*-retinal to ATR in the retinal-binding site of the mutant opsin, we employed a retinal analogue with the 11-*cis* bond locked by a 7-membered ring (Ret-7, Fig. 2*A*, *inset*), which is incapable of undergoing isomerization at the $C_{11}=C_{12}$ bond. Ret-7 was reported to form a pigment readily, designated Rho-7, with opsin purified from bovine rod outer segments. Rho-7 displays a λ_{max} of 496 nm, close to that of Rho (500 nm) (18). Ret-7 is light-stable and inactive both *in vivo* and *in vitro* (19, 20) and can partially relieve desensitization after bleaching of the rod cells (19). These observations imply that the ground state of Rho-7 closely resembles that of native Rho, but it cannot be activated by light due to the locked $C_{11}=C_{12}$ *cis* double bond. The close similarity of Ret-7 and 11-*cis*-retinal in forming the ground state pigment and the inability of Ret-7 to isomerize, either thermally or photochemically, make it a satisfactory analogue for experiments to show that the dark activities observed in the Gly^{121} mutants do not involve thermal isomerization of 11-*cis* to ATR in the ligand-binding site.

We regenerated wild-type opsin as well as mutant opsins G121V and G121L with Ret-7. The pigments Rho-7, G121V-7, and G121L-7 were purified in DM. Fig. 2*A* shows the spectra of Rho and Rho-7, demonstrating the close resemblance of the two pigments. Rho-7 has a λ_{max} of 498 nm, slightly red-shifted from the value of 496 nm reported previously (18), but closer to that of Rho (500 nm). The absorbance at the λ_{max} for Rho-7 is slightly decreased compared with that of native Rho. The decrease can be accounted for by one or more of the following reasons: 1) a lowered ϵ value of Rho-7, 2) incomplete incorporation of Ret-7 in the retinal-binding site, or 3) lowered thermal stability of Rho-7 during purification.

G121L-7 was also very similar to G121L as shown in Fig. 2*B*. The λ_{max} of G121L-7 (470 nm) was slightly blue-shifted from that of G121L (475 nm), and the absorbance at the λ_{max} is also lowered compared with G121L. G121V-7 displayed a λ_{max} of 480 nm, close to the value of 478 nm for G121V. Therefore, for all the opsins tested, the λ_{max} values of the Ret-7 pigments were within a few nanometers of their respective native counterparts. Since the λ_{max} value of a pigment is very sensitive to the retinal-opsin interaction, this similarity suggests that Ret-7 binds wild-type as well as the mutant opsins tested in a fashion similar to 11-*cis*-retinal.

Purified pigments G121L-7 and G121L were tested in parallel for their ability to activate G_t in the dark (Fig. 2*B*). Both pigments displayed significant dark activity. Four independent experiments resulted in a mean of 2.1 ± 0.4 for the ratio of the dark activity of G121L-7 *versus* that of G121L. These results demonstrate that although Ret-7 cannot be converted to ATR either thermally or photochemically, it can still cause purified G121L-7 to activate G_t. These results strongly suggest that the 11-*cis* form of retinal, a strong inverse agonist for wild-type opsin and constitutively active mutants tested, functions as a partial agonist for the G121L mutant.

We demonstrated previously that loss-of-function phenotypes of the Gly^{121} mutants could at least partially be restored

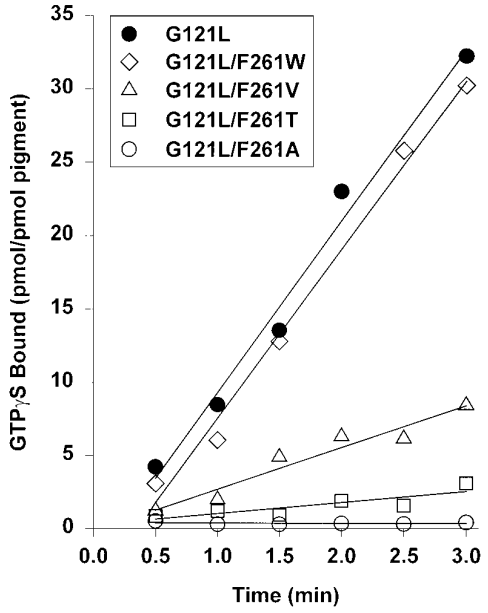

FIG. 3. **Light-independent G_t activation by mutant pigment G121L can be suppressed by a second-site mutation at position Phe^{261}.** The ability of mutant pigment G121L to catalyze GTPγS uptake by G_t in the dark was compared with that of double mutants G121L/F261W, G121L/F261V, G121L/F261T, and G121L/F261A. The suppression of the G121L dark activity was progressively lessened as the size of the amino acid side chain at position 261 was increased. Each mutant pigment was regenerated with 11-*cis*-retinal, purified, and assayed in DM detergent buffer. Pigment concentrations were determined based on the visible absorption at the λ_{max} (14, 15).

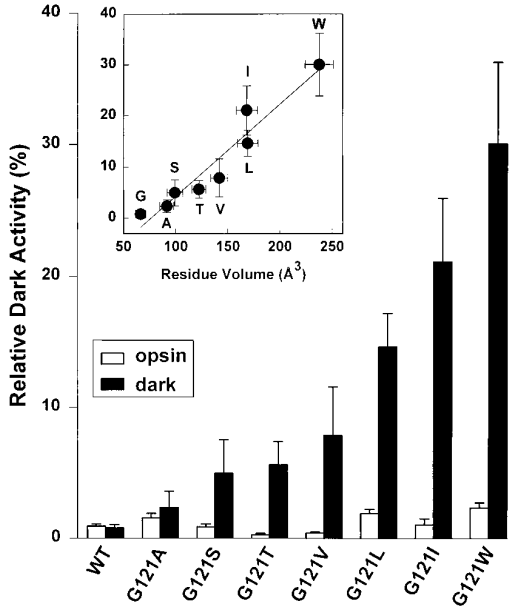

FIG. 4. **Light-independent G_t activation by a set of Gly^{121} replacement mutants.** For Rho (*WT*) and seven mutants, assays were carried out in transiently transfected COS cell membranes. Results are presented as relative dark activity, which is defined as the percentage of G_t activation measured under continuous illumination for the same sample in the presence of 11-*cis*-retinal. For pigment samples, COS cell membranes were incubated with 11-*cis*-retinal for 30 min before G_t activation assays were carried out. To determine opsin activities, 11-*cis*-retinal was not added to the membranes. Numerical values are presented in Table I. The *inset* demonstrates an apparent linear correlation between the level of relative dark activity and the volume of the residue at position 121. The residue volumes and errors are from Chothia (33). The linear correlation coefficient was 0.93, and the slope was 0.18%/Å^3.

by replacing Phe^{261} by alanine as a second-site mutation (15). Likewise, the high level of dark activity of mutant G121L could be reversed by second-site Phe^{261} mutation (Fig. 3). A series of Phe^{261} second-site mutants was tested for the ability to suppress the high dark activity of G121L. Mutants G121L/F261A, G121L/F261T, G121L/F261V, and G121L/F261W were prepared. While the F261A mutation completely suppressed the dark activity of G121L, F261W did not show any effect. F261T and F261V exhibited intermediate effects. The effect of the second-site mutation correlates well with the volume of the residue at position 261, suggesting a possible steric nature of the rescuing effect. Other double mutants tested (G121L/I219A, G121L/M257A, G121L/V258A, and G121L/W265Y) did not display such a rescuing effect (not shown). These second-site mutations failed to rescue other phenotypes of the G121L mutant as well (15).

The dark activity of the Gly^{121} mutants in the presence of 11-*cis*-retinal was also tested in COS cell membranes. This assay has the advantage of being able to characterize retinal-opsin complexes that are not stable in detergent and to measure constitutive activity of mutant opsins. Each of the Gly^{121} mutants displayed significant dark activity in the presence of 11-*cis*-retinal under conditions where Rho showed essentially no activity (Fig. 4). The ability of opsin alone to activate G_t (opsin activity) is also presented. Each of the Gly^{121} mutants displayed higher G_t activation in the presence of 11-*cis*-retinal, and none of them displayed significant constitutive activity. Under these conditions 11-*cis*-retinal is a partial agonist for each of the Gly^{121} mutant opsins (Table I).

The level of dark activity correlated linearly with the size of the residue substituted at position 121 (Fig. 4, *inset*). This size-dependent behavior is reminiscent of the linear correlation between the size of the residue at position 121 and the λ_{max} value in wavenumbers of the same set of Gly^{121} mutants described earlier (14). Together these results suggest a linear correlation between the λ_{max} value in wavenumbers and the level of the dark activity of these mutants in COS cell membranes. The size dependence of the blue shifts and the dark activities of the Gly^{121} mutants suggest that both phenotypes result from an increased steric interaction involving position 121.

The level of the dark activity of Gly^{121} mutants is always higher in COS cell membranes than that observed in DM-purified pigments. This suggests that the conformation of the receptor giving rise to the dark activity may not be as stable under conditions of detergent solubilization and purification. The ability of the second-site mutations to suppress the dark activity of the Gly^{121} mutants was also tested in COS cell membranes. The opsin activity and dark activity of Phe^{261} single mutants F261A, F261T, F261V, and F261W and their respective G121L double mutants G121L/F261A, G121L/F261T, G121L/F261V, and G121L/F261W were measured (Table I). Since many mutants have significant constitutive activity, the best measure of the partial agonist activity of 11-*cis*-retinal is the difference between the dark and opsin activities (Table I). None of the Phe^{261} single mutants displayed significant dark activity. The F261A replacement reduced the dark activity of G121L/F261A to 4.4% from 14.6% observed in G121L. G121L/F261A displayed significant constitutive activity (9.4%), which could be partially suppressed by the presence of 11-*cis*-retinal. Therefore, 11-*cis*-retinal acts as an inverse agonist for G121L/F261A mutant opsin (as it does for wild-type opsin), rather than as a partial agonist. The double mutant G121L/F261A not only has decreased dark activity compared with G121L, but the second-site mutation reverts the nature of the agonism of the 11-*cis*-retinal ligand.

G121L/F261T also displayed significant constitutive activity

TABLE I
Opsin and 11-cis-retinal dark activities in COS cell membranes
Values are given as mean ± S.E. (n), $n = 3$ unless otherwise specified.

Opsin	Opsin activity[a]	Dark activity[a]	Dark minus opsin	Agonism of 11-*cis*-retinal[b]
	%	%		
Wild-type	0.9 ± 0.2 (5)	0.8 ± 0.3 (5)	−0.1	iA
E113A	20.9 ± 5.8	3.6 ± 1.4	−17.3	iA
G121A	1.6 ± 0.3	2.3 ± 1.3	+0.7	pA
G121S	0.9 ± 0.2	5.0 ± 2.6	+4.1	pA
G121T	0.3 ± 0.1	5.6 ± 1.8	+5.3	pA
G121V	0.4 ± 0.1	7.8 ± 3.7	+7.4	pA
G121L	1.9 ± 0.3	14.6 ± 2.5 (5)	+12.7	pA
G121I	1.0 ± 0.4	21.1 ± 4.8	+20.1	pA
G121W	2.3 ± 0.4	30.0 ± 6.2 (6)	+27.7	pA
E113A/G121L	35.1 ± 8.5	46.5 ± 14.5	+11.4	pA
F261A	1.0 ± 0.2	1.7 ± 0.1	+0.7	iA/AN
G121L/F261A	9.4 ± 3.6	4.4 ± 1.1	−0.5	iA
F261T	1.9 ± 0.5	1.8 ± 1.1	−0.1	iA/AN
G121L/F261T	13.3 ± 1.8	12.4 ± 4.1	−0.9	AN
F261V	7.6 ± 1.6	2.5 ± 0.4	−5.1	iA
G121L/F261V	47.9 ± 5.3	27.0 ± 8.2	−20.9	iA
F261W	0.2 ± 0.0	0.5 ± 0.1	+0.3	iA/AN
G121L/F261W	4.8 ± 1.2	12.4 ± 4.1	+7.6	pA

[a] Results are presented as the percentage of G_t activation measured under continuous illumination in the presence of 11-*cis*-retinal for the same sample. Opsin activity and dark activity are measured in the absence and presence of 11-*cis*-retinal, respectively.
[b] Possible states of agonism for 11-*cis*-retinal include: iA, inverse agonist; pA, partial agonist; AN, antagonist; iA/NA, unable to distinguish between iA or NA because the opsin activity is very low.

(13.3%), which in this case is not significantly decreased in the presence of 11-*cis*-retinal (12.4% dark activity). From the high level of light activation of this mutant (15), we know that the chromophore is incorporated into its binding site; therefore, 11-*cis*-retinal does not shift the equilibrium between the active (R*) and inactive forms (R) of the aporeceptor and functions as an antagonist for this mutant (10, 11). Although the dark activity of G121L/F261T is similar to that of G121L, the difference between dark and opsin activities is greatly reduced. Therefore, the second-site F261T mutation is capable of rescuing the dark activity of G121L, changing 11-*cis*-retinal from a partial agonist to an antagonist.

G121L/F261V exhibits a very high level of constitutive activity (47.9%) that can be partially suppressed (27.0% dark activity) by 11-*cis*-retinal, which functions as an inverse agonist. Although the apparent dark activity of G121L/F261V is higher than that of G121L, F261V is still capable of suppressing the G121L dark activity judging by the difference of dark activity minus opsin activity of the double mutant. It is clear that a second-site mutation of Phe^{261} by a smaller residue can rescue the abnormal partial agonism of 11-*cis*-retinal in the Gly^{121} mutants. G121L/F261W displays essentially the same dark activity as G121L, which is expected since Phe^{261} is replaced by a larger residue.

Several other positions were also tested for their ability to rescue the dark activity of G121L mutants. The dark activities for double mutants E113A/G121L (46.5%), G121L/I219A (34.0%), G121L/M257A (78.9%), G121L/V258A (14.2%), and G121L/W265Y (32.5%) are all greater than their respective opsin activities; furthermore, they are equal to or greater than that of G121L. These observations demonstrate that among all the second-site replacements tested (Glu^{113}, Ile^{219}, Met^{257}, Val^{258}, Phe^{261}, and Trp^{265}) only substitution by a smaller residue on Phe^{261} could suppress the high dark activity of the Gly^{121} mutants.

The previous result that the constitutive activity of mutant opsin E113Q can be suppressed by 11-*cis*-retinal (9) was reproduced with mutant opsin E113A (Table I). Likewise M257A, which was also reported to be constitutively active (15), could be suppressed by 11-*cis*-retinal (not shown). Interestingly, for all the mutants tested with opsin activities ranging from 0.2 to 47.9%, ATR always served as an agonist (14, 15). The activity of each mutant in the presence of ATR was also invariably higher than its dark activity, suggesting that ATR is always a more potent agonist than 11-*cis*-retinal.

DISCUSSION

Here we report that the natural inverse agonist, 11-*cis*-retinal serves as a partial agonist in Rho mutants. Similar results have been reported for other G protein-coupled receptors. The mutations D113E in the β-adrenergic receptor (21), N111A in the AT_{1A} angiotensin II receptor (22), and L286A in the D1 dopamine receptor (23) changed the receptor specificity such that antagonist or inverse agonist ligands displayed partial agonist activity. These mutations occur at the positions equivalent to Ala^{117} and Gly^{120} on TM helix 3 and Leu^{266} on TM helix 6 of Rho, respectively. In addition, in the seven TM phototaxis receptor, sensory rhodopsin I, the D201N mutation converted the normally attractant signal of orange light to a repellent signal (24).

Because the opsin experiments are complicated by the potential thermal conversion between the inverse agonist 11-*cis*-retinal and the agonist ATR, we used the locked retinal analogue to show that retinal in its 11-*cis* isomeric state can activate Gly^{121} mutants. Another retinal analogue, 13-demethyl-retinal, was also reported to activate opsin in its 11-*cis* form, although the activity decreased over time (25). The dark activity of G121L opsin in the presence of 11-*cis*-retinal does not change significantly over a period of up to 4 h (not shown). This suggests that the nature of receptor activation in these two cases may be different. The ability of Rho to maintain an active conformation even with 11-*cis*-retinal in the binding pocket was also demonstrated in a set of experiments in which photo back-conversion of ATR to 11-*cis*-retinal was achieved without loss of activity (26).

Rho is a unique member of the G protein-coupled receptor family in that its natural agonist (ATR) is converted from an inverse agonist (11-*cis*-retinal) through photolysis. It is necessary that such conversion occurs in the retinal-binding site, since exogenous ATR can only partially activate opsin (14% of the light activity of Rho) (14) and apparently does not form a covalent bond with opsin (27). The isomeric state of the ligand dictates the receptor activation state. In wild-type opsin the ligand-binding site strictly prefers 11-*cis*-retinal over ATR. In Gly^{121} mutants, bulky substitutions at position 121 impose progressively increased steric interactions between retinal and opsin (14). The partial agonist activity of 11-*cis*-retinal in the Gly^{121} mutants is also linearly correlated with the size of the substituting residue (Fig. 4, *inset*). Site-directed spin-label and biochemical studies of Rho reveal that the formation of R* involves rigid body movement of TM helices 3 and 6 away from the ligand-binding site (28, 29). We and others (14, 15, 29–32) have shown previously that the retinal chromophore is located between TM helices 3 and 6, directly interacting with Gly^{121} and Phe^{261} on these helices. It is possible that the increased steric interaction between retinal and a Gly^{121} mutant opsin causes movements of the helices similar to those forming R*, although to a much smaller extent, resulting in the partial activity in the dark. The increased steric interaction can be relieved by substituting Phe^{261} with a residue with a smaller side chain, which reverts the partial agonism of the 11-*cis*-retinal ligand (Fig. 3).

We have shown previously that the ligand-binding site of G121L mutant opsin is altered such that it incorporates ATR faster than 11-*cis*-retinal. ATR can also directly activate G121L

opsin at much higher efficiency than wild-type opsin (56%) (14). In addition, the large blue shift (λ_{max} = 475 nm) and the high hydroxylamine reactivity of the G121L mutant imply that the chromophore-opsin interaction is significantly altered. It is possible that 11-*cis*-retinal in the G121L mutant binds in an orientation similar to that of ATR agonist and functions analogously, albeit less efficiently. This possible mechanism of partial agonist activity of 11-*cis*-retinal in the Gly^{121} mutant opsins is not mutually exclusive from the one discussed above in which steric effects allow 11-*cis*-retinal to facilitate helix movements normally associated with receptor activation.

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