A Naturally Occurring Mutation of the Opsin Gene (T4R) in Dogs Affects Glycosylation and Stability of the G Protein-coupled Receptor*

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Rho (rhodopsin; opsin plus 11-cis-retinal) is a prototypical G protein-coupled receptor responsible for the capture of a photon in retinal photoreceptor cells. A large number of mutations in the opsin gene associated with autosomal dominant retinitis pigmentosa have been identified. The naturally occurring T4R opsin mutation in the English mastiff dog leads to a progressive retinal degeneration that closely resembles human retinitis pigmentosa caused by the T4K mutation in the opsin gene. Using genetic approaches and biochemical assays, we explored the properties of the T4R mutant protein. Employing immunoaffinity-purified Rho from affected RHO* cis-Rho dog retina, we found that the mutation abolished glycosylation at Asn \(^2\), whereas glycosylation at Asn \(^{15}\) was unaffected, and the mutant opsin localized normally to the rod outer segment. Moreover, we found that T4R Rho* lost its chromophore faster as measured by the decay of meta-rhodopsin II and that it was less resistant to heat denaturation. Detergent-solubilized T4R opsin regenerated poorly and interacted abnormally with the G protein transducin (Gt). Structurally, the mutation affected mainly the “plug” at the intradiscal (extracellular) side of Rho, which is possibly responsible for protecting the chromophore from the access of bulk water. The T4R mutation may represent a novel molecular mechanism of the pathology caused by this mutant has not been elucidated (16). The prediction is that such a mutation will prevent the glycosylation at Asn \(^2\), one of the two glycosylation sites in opsin. The effects of glycosylation on the retina and Rho functioning have been investigated previously (11, 17–20). Tunicamycin, which inhibits the biosynthesis of N-acetylgalcosaminylphosphorylpolysipeinol and thus prevents the formation of the Asn-linked oligosaccharides of glycoproteins, blocks the glycosylation of opsin in vitro, but only slightly decreases the polyepitope synthesis and intracellular transport of opsin (18, 19). When expressed in a heterologous system, the T4K mutant displays increased mobility in SDS-PAGE experiments, suggesting that one or both sites (Asn\(^2\) and/or Asn\(^{15}\)) have not been glycosylated. The T4K mutant also regenerates poorly with 11-cis-retinal and displays low light-dependent activation of the rod photoreceptor G protein transducin (Gt) (11). The N2Q mutant has normal properties, whereas non-glycosylated Rho is ineffective in the G protein activation assay (19). After deglycosylation by peptide N-glycosidase F, Rho has spectral properties in the dark; and after bleaching, it retains properties similar to those of the native protein (20). When glycosylation at Asn\(^2\) and Asn\(^{15}\) is prevented, Rho inserts properly into the phospholipid bilayer and can be regenerated with 11-cis-retinal (20, 21). It also is properly synthesized and intracellularly transported (17), but its ability to carry out the light-dependent activation of G, is diminished (19).

An opportunity to study the mechanism of retinitis pigmentos...
tosa (RP) \(^1\) pathogenesis was presented when the T4R mutation was discovered in the English mastiff dog. The affected dogs display a dramatically slowed time course for recovery of retinal degeneration.

### Experimental Procedures

**Animals**—All dogs (3–9 months old) studied were from a research colony of mixed-breed dogs maintained at the Retinal Disease Studies Facility (Kennett Square, PA). **Rho** \(^{T4R}\) and **Rho** \(^{T4R/T4R}\) dogs derive their mutant **Rho** allele from English mastiff dogs affected with autosomal dominant progressive retinal atrophy as described previously (22, 23). RPE65/− and RPE65/−, RPE65/−/−, and RPE65/−/−/− dogs derive their mutant allele, a 4-bp deletion in the canine RPE65 (retinal pigment epithelium protein of 65 kDa) gene, from a bSocial dogs affected with the canine homolog of Leber’s congenital amaurosis (24, 25). All dogs used in this study were tested to determine their genotype at either or both the **Rho** (22, 23) and RPE65 (25) loci as described previously. To produce **Rho** \(^{T4R}\), RPE65/−/− double mutant dogs, an initial crossing was undertaken between an affected female from the **Rho**/−/− and RPE65/−/−/− strain (genotype **Rho** \(^{T4R}\) \(\times\) **Rho** \(^{T4R}\) \(\times\) RPE65/−/−) and a heterozygous affected male from the **Rho**/−/− strain (genotype **Rho** \(^{T4R}\) \(\times\) **Rho** \(^{T4R}\) \(\times\) RPE65/−/−). One of their female progeny genotyped as **Rho** \(^{T4R}\) \(\times\) **Rho** \(^{T4R}\) \(\times\) was then backcrossed to an RPE65/−/− male, yielding, among others, **Rho** \(^{T4R}\) \(\times\) RPE65/−/− double mutant individuals.

Whole retinae were dissected from eyes enucleated immediately postmortem from dogs that had been dark-adapted for at least 15 h. The retinae were then immediately frozen in liquid nitrogen and maintained under cryogenic conditions until analyzed. All such procedures were undertaken under dim red illumination.

**Materials**—11-cis Retinal was a gift from Dr. R. K. Crouch (University of South Carolina) through a contract with the National Institutes of Health. **N-Desetyl-N2-maltolose** (DM) was purchased from Anatech Inc. (Maumee, OH). GTP \(\gamma\)S was purchased from Sigma. Trypsin was purchased from Princeton Separation, Inc. (Adelphia, NJ). Monoclonal antibodies against the **Rho** C terminus was purchased from the National Cell Culture Center (Minneapolis, MN). Monoclonal antibody B6-30N raised against the **Rho** N terminus was a generous gift from Dr. P. A. Hargrave (University of Florida). The anti-Gt monoclonal antibody was a generous gift from Dr. H. E. Hamm (Vanderbilt University Medical Center). The anti-Gt \(\beta\) polyclonal antibody was a generous gift from Dr. O. G. Kissel (St. Louis University School of Medicine). The anti-Rho C-terminal peptides were purchased from United Biochemical Research, Inc. (Seattle, WA). The amount of purified bovine Gt was measured using a 2-D Quant kit (Amersham Biosciences). The hexyl-agaroose resin was purchased from MP Biomedicals Inc. (Aurora, OH). Other resins and columns for chromatography were purchased from Amersham Biosciences.

**Purification of Rho**—Purified anti-Rho C terminus antibody ID1 (24) was immobilized on CNBr-activated Sepharose 4B (27), and a 4.6 × 12-mm column was packed with 2 mg of antibody ID1/mL of Sepharose beads. All procedures employing Rho or retinoids were performed under dim red light unless mentioned otherwise. The dog retinae were homogenized in buffer containing 137 mM NaCl, 5.4 mM Na\( _2\)HPO\( _4\), 2.7 mM KCl, and 1.8 mM KH\( _2\)PO\( _4\) (pH 7.5) with a glass-to-glass homogenizer. Soluble proteins in the supernatant were removed by centrifugation at 12,000 \(\times\) g for 5 min, and the pellet was washed extensively with a solution of 50% MeOH and 10% AcOH. The washed gel pieces were dehydrated with CH\( _3\)CN and digested for 24 h with 0.5 \(\mu\)g of trypsin in 100 \(\mu\)l of 50 mM NH\( _4\)HCO\( _3\) at 40 °C. After digestion, the gel pieces were washed with 50 mM NH\( _4\)HCO\( _3\), dehydrated with CH\( _2\)CN, and dried again. The dried gel pieces were alkylated with 50 mM iodoacetamide in 50 mM NH\( _4\)HCO\( _3\) at room temperature for 20 min. The dried gel pieces were washed overnight with a solution of 50% MeOH and 10% AcOH. The washed gel was stained with a mixture of 0.02% Coomassie Blue R-250, 20% MeOH, and 10% AcOH and then destained with a solution of 50% MeOH and 10% AcOH. The protein bands corresponding to T4R \(\times\) Rho C terminus were excised into small pieces (1.0 × 1.0 mm) and extensively washed with a solution of 50% MeOH and 5% AcOH. After being dehydrated with CH\( _2\)CN and dried in a SpeedVac, the gel pieces were treated for 40 min with 10 mM dithiothreitol (DTT) in 50 mM NH\( _4\)HCO\( _3\) at 40 °C. The solution was removed, and the gel pieces were rinsed with 50 mM NH\( _4\)HCO\( _3\), dehydrated with CH\( _2\)CN, and dried again. The dried gel pieces were alkylated with 50 mM iodoacetamide in 50 mM NH\( _4\)HCO\( _3\) for 40 min in the dark at 40 °C. After removal of the solution, the gel pieces were incubated with two rounds of 50 mM NH\( _4\)HCO\( _3\) at room temperature for 10 min each time. Finally, the gel pieces were dehydrated with CH\( _2\)CN and digested for 24 h with 0.5 \(\mu\)g of trypsin in 100 \(\mu\)l of 50 mM NH\( _4\)HCO\( _3\) at 37 °C. To digest the digested peptides, the solution was removed, and the digested gel pieces were incubated with another 100 \(\mu\)l of 50 mM NH\( _4\)HCO\( _3\) at room temperature for 10 min. The incubation was repeated with two rounds of 50 mM NH\( _4\)HCO\( _3\), 10% AcOH and 90% CH\( _2\)CN, 90% CH\( _2\)CN and 10% AcOH, and 90% MeOH and 10% AcOH.

### Electrophoresis and Immunoblotting

**Protein separation** was performed on 10% SDS-polyacrylamide gels. Coomassie Blue R-250 staining, silver staining, and immunoblotting (Immobilon-P polyvinylidene difluoride, Millipore Corp.) were carried out according to standard protocols. Antibodies ID4 (26) and B6-30N (27) were used to detect the corresponding protein bands of Rho. The purified Rho concentration was determined using a UV-visible spectrophotometer.

**Deglycosylation of Rho**—Peptide N-glycosidase F (New England BioLabs Inc.) was used for the Rho deglycosylation experiments. In brief, ~20 \(\mu\)g of immunopurified-Rho was first denatured (according to the manufacturer’s protocol) and incubated with 100 units of peptide N-glycosidase F at 37 °C for 14 h. Because deglycosylated Rho has a faster mobility on SDS-polyacrylamide gel (21), the samples were then immunoblotted with antibody ID4 to confirm the completion of deglycosylation.

**Quantification and Stability of Rho**—Quantification of dog Rho was carried out spectrophotometrically. Whole dog retinas were homogenized with a glass-to-glass homogenizer. After centrifugation at 14,000 \(\times\) g for 5 min, the pellet was solubilized in buffer containing 1% DM, 10 mM bis-tris-propane, and 150 mM NaCl (pH 7.5). The solubilized mixture was centrifuged at 125,000 \(\times\) g for 20 min, and one-fourth of the supernatant was used for quantification of the Rho concentration by UV-visible spectroscopy. To determine the stability of the Rho in the supernatant was used for quantification of the Rho concentration by UV-visible spectroscopy. To determine the stability of the Rho in the supernatant, the absorbance spectra of freshly purified Rho samples were measured at 37 °C in the absence or presence of 20 mM NH\( _4\)OH (pH 7.0). The absorbance was recorded at 504 nm until it dropped below 30% of the initial reading. Standard deviations were calculated from three sets of data from parallel experiments.

**Retinoid Analysis**—Retinoids were extracted and derivatized as described previously (28–33). Samples were analyzed by normal-phase HPLC (Beckman UltraspHERE-Si HP1100 column, 5 \(\mu\)m, 4.6 × 250 mm) using an isocratic solvent system consisting of 0.5% (v/v) ethyl acetate in hexane for 15 min, followed by 4% ethyl acetate in hexane for 60 min at a flow rate of 1.4 ml/min at 20 °C. The diode array UV-visible detector was set at 325 nm. Data were analyzed with Hewlett-Packard Chemstation A.06.03 software.

**T4R Mutant of Rhodopsin**

1. The abbreviations used are: RP, retinitis pigmentosa; DM, n-dodecyl \(\beta\)-N-maltoside; GTP\(\gamma\)S, guanosine 5\(’\)-O-(3-thiotriphosphate); bis-tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; HPLC, high performance liquid chromatography; DTT, dithiothreitol; LC-MS/MS, liquid chromatography-tandem mass spectrometry; WT, wild-type; Meta II (Rho\(\gamma\)), metR-phase rhodopsin II (photoactivated rhodopsin); IOS, rod outer segments; MOPS, 4-morpholinepropanesulfonic acid.

The \(\beta\)Ar Mutant of Rhodopsin
dissolved in solvent A to a final peptide concentration of 1 μM for LC-MS/MS analysis.

Mass Spectrometry—Analysis of the tryptic N-terminal peptide Ac-MNGR (MH+1, m/z 519.2354) was performed by microcolumn electrospray LC-MS/MS. The mass spectrometer (PE-Sciex API QSTAR PULSAR i Q-TOF) was coupled with a nebulization-assisted electrospray ionization source (PE-Sciex). The house-made microcolumn (250 μm × 15 cm) was prepared from fused silica tubing packed with Zorbax SB-C18 (4 μm, 80 Å) using an inline microfilter as the column outlet frit. An Agilent 1100 series HPLC capillary pump was used to deliver a flow rate of 4 μL/min with a gradient of 100% solvent A (0.2% AcOH) and 0% solvent B (90% CH3CN and 0.2% AcOH) for 18 min and then gradually to 0% solvent A and 100% solvent B for another 50 min. Throughout the gradient, the mass spectrometer was programmed to monitor m/z in the range of 500–550 with a +1 charge only, followed by MS/MS (unit resolution) with three different preset values of collisional energy for each selected precursor ion. Similar results were obtained in two independent experiments.

Photosensitivity and Regeneration of the Visual Pigment—UV-visible absorption spectra of freshly purified dog Rho were first measured at 20 °C. The samples were then bleached at a 20-cm distance (to avoid overheating the sample) with a 60-watt light bulb for 10, 20, 30, and 300 s. Alternative bleaching was done using a long-pass wavelength filter (>490 nm). The results were comparable under both bleaching conditions. The UV-visible absorption spectra of the bleached samples were immediately measured after each bleaching. In the experiments without NH2OH, H2SO4 was added after bleaching the sample for 5 min to adjust the final pH to 1.9 to trap the retinylidene Lys296. In another set of experiments, neutral NH2OH was added to reach a final concentration of 20 mM before any bleaching was conducted. In the regeneration experiments, ~10 μg of wild-type (WT) or T4R Rho solubilized in 1% DM or 2% digitonin was bleached thoroughly for 2 min with a Fiber-Lite illuminator at a distance of 15 cm. Next, 11-cis-retinal was added under dim red light, and the formation of Rho was measured immediately afterward. The final ratio of 11-cis-retinal to Rho was 50:1, which enabled fitting of the data to a pseudo first-order reaction (P<sub>met</sub> < 0.0001, P<sub>met</sub> < 0.0008). The rate constants calculated for dog WT and T4R Rho were 0.00029 and 0.00001 s⁻¹, respectively.

Limited Proteolysis—Freshly purified dog WT and T4R Rho were quantified by UV-visible spectroscopy. Half of the samples were bleached by illumination with a Fiber-Lite illuminator for 5 min at room temperature in the presence of 20 mM neutral NH2OH. Both Rho and the bleached opsin samples were digested at a 200:1 molar ratio of Rho to tosylphenylalanyl ketone-treated trypsin (Worthington) in buffer containing 137 mM NaCl, 5.4 mM Na2HPO4, 2.7 mM KCl, and 1.8 mM KH2PO4 (pH 7.5) for 5, 15, 30, or 45 min. The digestion was terminated by the addition of denaturing loading buffer (350 mM Tris-HCl, 350 mM SDS, 30% (v/v) glycerol, 600 mM dithiothreitol, and 175 mM bromphenol blue), and the resulting mixture was immediately used for SDS-PAGE or immunoblot analysis.

Rates of meta-Rhodopsin II (Meta II) Decay—All measurements were performed with 0.1 nM dog Rho in a solution of 0.1% DM, 10 bis-tris propane, and 100 mM NaCl (pH 6.0), favoring the formation of Meta II. A PerkinElmer Life Sciences LS 50B luminescence spectrophotometer was used to measure the increase in intrinsic Trp fluorescence due to hydrolysis of the protonated Schiff base and release of all-trans-retinal.
from Rho (32–34). Immunoaffinity-purified Rho was bleached with a Fiber-Lite illuminator for 15 s from a distance of 15 cm, immediately followed by fluorescence measurements. A thermostat was applied to stabilize the temperature of the cuvette at 20 °C during the measurement. Fluorometer slit settings were 2.5 μm at 295 nm for excitation and 8 μm at 330 nm for emission. Both fitted curves have R² values >0.99.

**Purification of Gα**—Gα was extracted from fresh bovine rod outer segment (ROS) membranes. ROS from 200 bovine retinas were homogenized in 80 ml of ice-cold water. The membrane suspension was centrifuged at 45,000 g for 30 min, and the supernatant was collected. This extraction was repeated twice. The combined supernatant was centrifuged at 45,000 × g for 30 min to remove ROS membrane contamination. The final supernatant was adjusted to contain 10 mM MOPS, 2 mM MgCl₂, 1 mM DTT, and 0.5 mM benzamidine (pH 7.5). This solution was then loaded onto hexyl-agarose resin (10 × 55 mm) equilibrated with 100 mM NaCl and 1 mM DTT. The protein concentration of each fraction was determined using a PerkinElmer LS 50B luminescence spectrophotometer (Varian Inc., Walnut Creek, CA), and the fractions were then analyzed by SDS-PAGE and immunoblotting as described above.

The fluorescence assay of Gα activation, the ratio of Gα to Rho was ~10:1, with Gα at a concentration of 250 nM and Rho at 25 nM, within the linear range of fluorescence change and protein concentrations. The sample was bleached for 15 s using a Fiber-Lite covered with a long-pass wavelength filter (>490 nm), followed by a 10-min incubation (if not indicated otherwise) with continuous low-speed stirring. The intrinsic fluorescence increase from Gα, was measured with the PerkinElmer Life Sciences LS 50B luminescence spectrophotometer employing excitation and emission wavelengths at 300 and 345 nm, respectively, as described (32–36). No signals from Rho in the absence of Gα were detected in the control experiment.

**Histology and Immunocytochemistry**—Canine retinas were prepared for morphological studies either by a triple fixation protocol (37) before embedding in plastic or by 4% paraformaldehyde fixation for cryosectioning and immunocytochemistry after optimal cutting temperature compound embedding. For immunocytochemical studies, sections from...
The T4R Mutant of Rhodopsin

RESULTS

Mutation T4R—The T4R opsin mutation affects the region that is sequestered in the intradiscal space (Fig. 1A). The structures of WT and T4R Rho appear to be almost identical, as predicted from molecular dynamics (Fig. 1A). The T4R mutation does not influence the anchoring of Rho in the membrane because residue 4 is located very close to the intradiscal tip of Rho and is far from the membrane. Instead, Arg⁴ points its charged group outside Rho and interacts with the adjacent Glu⁵. The intradiscal surface in WT Rho is mostly negatively charged, and once mutated, the additional positive charge of Arg⁴, or differences in protein folding in the vicinity of this region prevent glycosylation at Asn² (Fig. 1A, upper middle panels, red). The extracellular region of Rho is highly conserved among mammalian opsins, and a number of pathogenic mutations in mammals leading to autosomal dominant RP have been identified (Fig. 1B). Two sets of mutations will affect glycosylation at Asn² and Asn⁴ either by substitution of these residues with a residue that cannot be glycosylated or by elimination of consensus sequences that are essential for recognition by endoplasmic reticulum oligosaccharyltransferase. It is unclear how mutations in the intradiscal region affect oligomerization of Rho (Refs. 40–43; see also Ref. 4).

Mutant Opsin Accelerates Degeneration—Dog T4R opsin is more toxic to photoreceptor cells than Rho, as shown in the following genetic experiment. The absence of the retinal chro-
mophore correlates with an increased toxicity of the protein. The fundus of the left eye of an 8-month-old affected RHO\textsuperscript{T4R/T4R} dog appeared to be within normal limits, with uniformly normal morphology and no ophtalmoscopic evidence of retinal degeneration (Fig. 2, A1-A3). It should be noted that older animals (>10 months) begin to exhibit severe retinal degeneration, initially in distinct topographic regions that later become more generalized (22). Fundus photomontages of the left eye of a 6.5-month-old RHO\textsuperscript{T4R/T4R} dog, which could not produce the chromophore because of mutation in the RHOT\textsuperscript{A1-A3} gene (24, 25, 44), displayed hyperreflectivity of the tapetal fundus (Fig. 2B1), moderate vascular attenuation, and changes characteristic of outer retinal thinning and mid-stage disease. This fundus appearance reflects the photoreceptor degeneration present uniformly throughout the double mutant retina, as shown in histological sections in Fig. 2 (B2 and B3).

**Fig. 4. Glycosylation status of T4R Rho.** A, glycosylation status of extracted and purified Rho from dogs of different genetic backgrounds with respect to Rho mutation. The Coomassie Blue-stained SDS-polyacrylamide gel shows supernatant solubilized in 1% DM (left) and immunoaffinity-purified Rho (right) from WT, RHO\textsuperscript{T4R/T4R}, and RHO\textsuperscript{T4R/T4R} dog retinas before and after deglycosylation with peptide N-glycosidase F (PNGase F) was detected with anti-Rho C terminus antibody ID4. C, immunoblot of Rho from different quadrants of WT, RHO\textsuperscript{T4R/T4R}, and RHO\textsuperscript{T4R/T4R} dog retinas. Similar results were obtained in three to five independent experiments. SN, superior nasal; ST, superior temporal; IN, inferior nasal; IT, inferior temporal.

**The T4R Mutant of Rhodopsin**

Retinal immunocytochemistry of a 2-month-old homozygous affected RHO\textsuperscript{T4R/T4R} dog showed normal morphology and ROS formation (Fig. 3A). The retina from a RHO\textsuperscript{T4R/T4R} dog was employed to isolate Rho by affinity chromatography or retinoids by analytical techniques in our studies. UV-visible absorption spectra of immunoaffinity-purified WT and T4R Rho showed almost identical spectra and A\textsubscript{280 nm}/A\textsubscript{500 nm} ratios of 1.70 and 1.72, respectively (Fig. 3B), suggesting that the mutation at this position does not affect the location of the intradiscal E2 loop of Rho, which comes close to the chromophore core (8, 45). As assessed by silver staining, WT Rho and its mutant were purified to apparent homogeneity (Fig. 3B, right panel). The amounts of Rho as measured by UV-visible spectroscopy of complete extracts from dog retina in WT, RHO\textsuperscript{T4R/T4R}, and RHO\textsuperscript{T4R/T4R} eyes were comparable (Fig. 3B, left panel, inset). Similarly, the quantities of 11-cis-retinal, identified by specific elution time and spectral properties (29, 30), in WT, RHO\textsuperscript{T4R/T4R}, and RHO\textsuperscript{T4R/T4R} retinal extracts were equal to values of 2.8, 2.65, and 2.5 nmol/eye, respectively (Fig. 3C, upper panel, inset). These results agree with the histological data. The elevated all-trans-retinyl esters in the RHO\textsuperscript{T4R/T4R} animal were within observed differences between individual dogs.

**T4R Rho Is Not Glycosylated at Asn\textsuperscript{2}**—The glycosylation status of T4R Rho in extracted and purified Rho was evaluated by SDS-PAGE (Fig. 4A) and immunoblotting (data not shown). Rho from WT retina displayed a single band and predictably had two glycosylation sites; Rho from the RHO\textsuperscript{T4R/T4R} retina had two bands indicating two forms of Rho, one with mobility equal to that of WT Rho and the other with higher mobility, suggesting only partial glycosylation. T4R Rho from RHO\textsuperscript{T4R/T4R} retina displayed a single band, most likely only singly glycosylated Rho. When Rho from WT, RHO\textsuperscript{T4R/T4R}, and RHO\textsuperscript{T4R/T4R} dogs was deglycosylated, the proteins had identical mobilities, which were somewhat faster than that of unmodified T4R Rho. All Rho samples moved faster after deglycosylation (Fig. 4B), suggesting that the Rho mutant is properly glycosylated at only a single site. Because the disease manifests regional differences in dog retina, we investigated whether there is a regional difference in the glycosylation. Immunoblotting of Rho from different quadrants showed very similar patterns (Fig. 4C), suggesting that there is no variability in glycosylation in the different sectors of the eye.

T4R Rho was in-gel digested with trypsin, and the released peptides were analyzed directly by LC-MS/MS. Interestingly, MS/MS data revealed that the standard and the sample gave m/z values of 520.1964 and 520.2368, respectively, corresponding to the Ac-MDGR peptide (calculated precursor ion monoiso-
The T4R Mutant of Rhodopsin

Fig. 5. LC-MS/MS analysis of tryptic N-terminal peptides. Shown are the results of analysis using the standard synthetic peptide Ac-MNGREGPGNYY (A) and immunoaffinity-purified T4R Rho (B). The expected product, Ac-MNGR, [M + H]+ at m/z 519.2354, was not observed, but instead, its deaminated product, Ac-MDGR, [M + H]+ at m/z 520.2194, was detected in both cases. Both MS/MS spectra of Ac-MDGR at m/z 520.2310 from the standard (A) and at m/z 520.1900 from T4R Rho (B) were nearly identical. The characteristic fragments for calculated m/z values are as follows: y4, 520.2184; y3, 347.1673; y2, 232.1404; y1, 175.1189; z2 or y2-NH3, 330.1408; z1 or y1-NH3, 158.0924; a1-H2O, 300.0970; and a1, 146.0640. The structure at the top shows the fragmentation patterns of this tetrapeptide. The analysis was reproduced in two independent experiments. The deamidated residue is shown in gray.

topic mass for [M + H]+ = 520.2184), which is the deaminated product of the authentic tryptic Ac-MNGR product (calculated monoisotopic mass for [M + H]+ = 519.2354). Both MS/MS spectra were almost identical, and the y ions series (y1–y4) could be fully observed together with some a ions and z ions or y ion-NH3 (Fig. 5, A and B). The strongest evidence of the presence of Ac-MDGR instead of Ac-MNGR came from y2 and y3, with the indication of the loss of a nominal mass of 115 Da (Asp). The observed fragmentation pattern is in agreement with the sequence Ac-MDGR. The same peptide was also identified with another trypsin from a different supplier (Worthington). Thus, this tryptic peptide is unexpectedly deaminated by trypsin. This does not change, however, the conclusion drawn from the analysis. Our results demonstrate clearly that the Asn residue in the second position of the T4R Rho mutant is not glycosylated.

T4R Rho Releases the Chromophore Faster than WT Rho—To study the release of retinal, immunoaffinity-purified WT Rho (Fig. 6, A and C) and T4R Rho (Fig. 6, B and D) were bleached in the presence and absence of NH2OH. No significant differences were observed. However, when the chromophore was trapped in the protonated Schiff base linkage with H2SO4, a smaller absorption peak was observed in the T4R Rho sample (~80% compared with WT Rho), suggesting that the chromophore after bleaching in T4R Rho was not as effectively retained in the binding site as in WT Rho (Fig. 6, A and B, blue traces).

We further measured the trapping of the chromophore in the binding site. The decay of Meta II was monitored (Fig. 7A) using a fluorescence assay that takes advantage of the increase in fluorescence when the chromophore leaves the binding pocket (32, 34). Meta II of T4R Rho decayed faster in the presence and absence of NH2OH than did Meta II of WT Rho (Fig. 7B). When the data were fitted to a first-order reaction, the time constants were calculated to be τ = 33.3 min for WT Rho and τ = 12.2 min for the mutant (Fig. 7C).

Dog mutant Rho was also less thermally stable than WT Rho (Fig. 8A). This difference in stability was diminished when a strong nucleophile (NH2OH) was added (Fig. 8B). The lack of stability against NH2OH is likely due to differences in the amino acid sequence within the chromophore-binding site of dog versus bovine Rho (Fig. 1B).

Regeneration in digitonin revealed another striking difference between dog WT and T4R Rho. In this detergent, T4R Rho regenerated slowly compared with WT Rho (Fig. 9). Similar differences were also observed in DM (data not shown). However, it should be noted that Rho regeneration took place in vivo in membranes (Fig. 3). The use of mild detergent may exacerbate the effect of mutation, uncovering more clearly the harmful effect of the mutation.

The Intradiscal Region of T4R Rho Is Less Structured—To investigate whether the intradiscal region is misfolded, limited proteolysis was employed. Trypsin cleaves the peptide bond at the C-terminal side of Arg or Lys when either is in an open configuration that can insert into the binding site of the protease. When Rho digestion was compared by SDS-PAGE and immunoblotting using anti-Rho C terminus and anti-Rho N terminus antibodies, major differences were observed (Fig. 10). WT Rho and T4R Rho lost only the C-terminal region as described previously (46) and were visualized by the anti-Rho N terminus antibody (doublet at ~36 kDa) (Fig. 10, B and E), as the C-terminal cleavage abolished recognition by the anti-Rho C terminus antibody (single band at ~36 kDa). In contrast to Rho, opsins were degraded much faster, as many low molecular mass species of the degraded proteins were observed. The N terminus of T4R opsin was digested much faster (Fig. 10F) than WT opsin (Fig. 10B). These differences could be a result of both the point mutation and the single glycosylation of T4R
opsin. These results suggest that the N terminus is primarily affected, leading to the loss of recognition by the anti-Rho N terminus antibody.

Rho*-Gt Binding and Activation—Next, the interactions between Gt and WT or T4R Rho* were investigated (Fig. 11, A–C). Once formed, the Rho*-Gt complex had a higher molecular mass than either Gt or Rho itself and migrated faster through the size exclusion chromatography column. When run through the column alone, Gt eluted in fractions 35–43 (data not shown). In the Gt binding experiments, together with either bleached WT Rho or T4R Rho, Gt was mainly present in fractions 33 and 35 (Fig. 11A, upper panels), indicating that the complexes were formed. However, Gt was also present in fractions 37 and above, co-eluted with a proportional amount of opsin. In the presence of GTPγS (non-hydrolyzable analog of GTP), the dissociation of Gt from WT or T4R opsin was observed as Gt shifted toward the later fractions (fractions 37–39) (Fig. 11, A and B, lower panels). Gt dissociated more slowly from the T4R opsin complex than from the WT opsin complex, given the observation of the relative lag in the elution of Gt with T4R opsin. This suggests that the possible conformational change in the T4R mutant may play a role in either stronger binding or slower release.

In the Gt activation assay, which monitors the rate of change in intrinsic fluorescence from Gα (47, 48), we obtained a much slower initial rate with T4R Rho* at either neutral or slightly acidic pH (Fig. 11C). WT Rho displayed a faster initial activation rate at neutral pH compared with slightly acidic pH, whereas the T4R mutant showed the opposite trend. The slower activation may be partially related to the faster decay of Meta II for the mutant. This observation was further supported by the experiment in which there was no GTPγS added until Rho* and Gt were incubated together for the measured relaxation time for the T4R mutant (τ = 12.2 min). There was little or almost negligible increase in the fluorescence of Gt upon addition of GTPγS when Rho* decayed (Fig. 11C, gray trace). These results are consistent with the relaxation time measured, since most of the Meta II is consumed by that time, Gt activation is expected to be much slower. The apparent initial rates calculated from each group of experiments were as follows: WT Rho at pH 7.5, k0 = 0.0026 s⁻¹; WT Rho at pH 6.0, k0 = 0.0019 s⁻¹; T4R Rho at pH 6.0, k0 = 0.0013 s⁻¹; T4R Rho at pH 7.5, k0 = 0.0005 s⁻¹; and T4R Rho at 800 s > τMeta,II at pH 7.5, k0 = 0.0003 s⁻¹. Together, the lack of activation as measured by the fluorescence assay and the differences in the gel filtration of the complexes indicate that the T4R Rho*-Gt interaction is aberrant.

DISCUSSION

In this study, we investigated the properties of T4R Rho isolated from affected canine retinas; Rho was synthesized, processed, and trafficked in its native host cells, the rod photoreceptors. The main points of our experiments include the following. 1) The T4R mutation eliminates sites of recognition at Asn2 by oligosaccharyltransferase throughout the dog retina, but it appears that Asn15 is glycosylated similarly compared with WT Rho. The lack of glycosylation at Asn2 does not prevent transport of the mutant protein to ROS and its regeneration. The full complement of Rho is found in the dark-adapted animals. 2) The deleterious effect of the T4R mutation is likely mediated by opsin rather than by Rho. This difference, evident in multiple biochemical assays, was confirmed by the
A genetic experiment combining the T4R Rho mutation with the RPE65 knockout mutation. In double mutant dogs, the RPE65 mutation leads to a lack of chromophore production and greatly accelerated retinal degenerative disease compared with the single mutant T4R retina. 3) Mutation at the N terminus affects the chromophore-binding site, as the release of the chromophore is accelerated, and in vitro regeneration with 11-cis-retinal is inhibited; the mutation also affects formation of the active Gt conformation and the prompt release of activated Gt. It is clear that solubilization of Rho in mild detergent exacerbates these differences compared with native T4R Rho in membranes. However, the visual process happens repeatedly in vivo, whereas in the purified system, it is a one-time process. The lower thermal stability of the mutant may further aggravate the inherited instability of opsin in vivo.

Dog Versus Bovine Rho—As expected, the general properties of canine Rho studied here and bovine Rho from a number of previous studies were similar. The glycosylation of dog WT Rho was uniform as judged by SDS-PAGE, which is similar to that of bovine Rho isolated from retina. No differences in regeneration or glycosylation were observed in different parts of the dog retina. In contrast, Rho expressed in heterologous systems was heterogeneously glycosylated. Without any amino acid changes on the cytoplasmic surface implicated in binding Gt (49, 50), dog WT Rho effectively stimulated bovine Gt. Bovine and dog Rho display similar bleaching properties, but due to nine changes within the chromophore-binding site (Fig. 1, lower panel), the half-life for chromophore release is 33.3 min for dog Rho (Fig. 7) compared with 15 min for bovine Rho (Ref. 32 and confirmed in our assay). Dog Rho was less stable with respect to NH2OH, again likely due to the differences in the amino acid residues within the chromophore-binding site.

In contrast, dog T4R Rho undergoes a different bleaching pathway, as the photoactivated mutant has a reduced ability to

![Fig. 7. Meta II decay of WT and T4R Rho. A, shown are the fluorescence emission spectra of WT Rho (left panel) and T4R Rho (right panel) before (black trace), immediately after (dark gray trace), and after thorough (light gray trace) photobleaching. B, the decay of WT Rho (black trace) and T4R Rho (red trace) Meta II was recorded with (lower panel) or without (upper panel) 10 mM NH2OH at pH 6.0. Similar results were obtained in five independent experiments. C, the data from B without NH2OH were fitted to a first-order reaction, which gives the relaxation times (t) of WT Rho (left panel) and T4R Rho (right panel) Meta II as 33.3 and 12.2 min, respectively. Similar results were obtained in five independent experiments.](image1)

![Fig. 8. Stability of WT and T4R Rho. The stability of Rho was measured as a change in the UV-visible absorption spectra at its absorption maximum at 37 °C without (A) or with (B) 20 mM neutral NH2OH. The results were plotted assuming 100% absorption at the initial point. Bovine Rho was used as a control for the NH2OH sample. Similar results were obtained in three independent experiments.](image2)
sylation of Rho at Asn15 is more important in signal transduction and may prevent glycosylation at Asn2. However, it is demonstrated by its normal palmitoylation, transport to the "plug" in maintaining the location of the chromophore and region. Both observations underscore the importance of the morphore appears to be destabilized by mutation in this general bound chromophore (14). Also, in the case of T4R, the chromophore affects the chromophore-binding site and somewhat surprising that the mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic face of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface.

activate Gt, likely in part because its photoproduct corresponding to Meta II decays approximately three times faster compared with WT Rho. The binding of Gt to T4R Rho photoprod-uct(s) appears also to form an aberrant nonproductive complex that could be detrimental to the physiology of the photoreceptors and ultimately lead to their demise. The nature of the deviant interaction between Gt and the T4R mutant has not been explained at the mechanistic level or correlated with cell physiology in this study.

Structural Considerations—The electrostatic potential of the N-terminal region in WT Rho is negative due to the presence of Glu. Introduction of Arg changes the positively charged distribution and may prevent glycosylation at Asn. However, it is somewhat surprising that the mutation on the intradiscal surface of the protein affects the chromophore-binding site and ultimately the cytoplasmic surface. We note that the P23H mutation on the intradiscal surface produces a hydrophilic channel in the interior of the protein that could accommodate water molecules and allow the hydrolysis of the covalently bound chromophore (14). Also, in the case of T4R, the chromophore appears to be destabilized by mutation in this general region. Both observations underscore the importance of the “plug” in maintaining the location of the chromophore and protecting it from hydrolysis (3).

In tissue culture, non-glycosylated opsin folds correctly as demonstrated by its normal palmitoylation, transport to the cell surface, and pigment formation with 11-cis-retinal (19). Similar to the T4R mutant described here, non-glycosylated Rho shows less efficient light-dependent activation of Gt. Using multiple substitutions that abolish glycosylation at Asn and Asn and reduced Gt activation, it was suggested that glycosylation of Rho at Asn is more important in signal transduction (19). Additionally, it was correctly deduced that the extracellular region is involved in the formation of a specific structure, at present known as the plug (8). Bleaching of glycosylated and non-glycosylated Rho results in similar photo-bleached products as determined by absorption spectra, but non-glycosylated Rho is inefficient in Gt activation (19). Similarly for the dog T4R Rho mutants, the lack of glycosylation at Asn and the introduction of the positive charge within this structurally sensitive region (Fig. 1A, upper and middle panels) are likely to be detrimental to protein stability and regeneration. Although bleaching appeared to be nearly normal, the signaling state of T4R Rho is clearly aberrant.

Congenital Night Blindness Versus RP Rho Mutants—Similar phenotypes as observed for dog T4R Rho could be more common and related to other Rho mutants characterized previously. Janz et al. (51) noticed an ion pair between Arg and Asp, near the highly conserved disulfide bond in the extracellular region. This ion pair is found in almost all vertebrate opsins (Ref. 51; reviewed in Ref. 52). The ion pair opsin mutants regenerate with 11-cis-retinal and couple to Gt. However, they are thermally unstable in the dark state because of rapid hydrolysis of the retinal Schiff base linkage, indicating that the extracellular E2 loop encompassing this particular ion pair is important for Rho stability. This observation suggests that the Asp mutant, observed in a subset of RP patients (53), may be thermally unstable and results in photoreceptor degeneration.

In addition, congenital night blindness mutants that are characterized by an open conformation of the chromophore-binding site for nucleophiles have been identified. For example, T94I opsin folds properly and binds 11-cis-retinal to form pigment, but T94I Rho displays reduced thermal stability, has a long lived Meta II photostate, and shows highly increased reactivity toward NH2OH in the dark compared with WT Rho (54, 55). In addition, it has been shown that T94I opsin is constitutively active (55). The abnormal function of this Rho mutant is likely related to the decreased thermal stability and opsin constitutive activity without protein misfolding. This property differentiates these and other congenital night blindness mutants (Refs. 55 and 56; reviewed in Ref. 57) from the structurally unstable mutants like T4R Rho described in this study or the previously mentioned RP Asp Rho mutant (53).

Interestingly, the most common RP mutant, P23H Rho, in addition to structural changes (14), is also less stable than the WT protein (15).

Potential Retinoid Supplementation and Lower Light Exposure May Counteract the RHO T4R Mutant—As both the mouse and human age, there is a decline in the rate of Rho regeneration (reviewed by Jackson et al. (58)), which ultimately leads to photoreceptor loss (59). This process likely extends to dogs as well. Therefore, as the animal ages, more and more of the T4R opsin that is unstable will be generated, leading to progressive destruction of ROS disc structure. The defect is relatively mild, and only when a certain threshold is reached is there a sectorial massive degeneration. Based on the results obtained with double mutant animals that lack the enzymatic pathway for retinoid production through the retinoid cycle (reviewed by McBee et al. (60)), it is likely that light will further exacerbate the problem in the presence of the T4R mutation (Fig. 2). This would also apply to the T4K mutation, and lower light exposure and dietary supplementation with the photoactive chrophophore may be therapeutic for RP patients with this class of mutations.

Interestingly, supplementation of 15,000 IU of vitamin A taken daily slows the progression of the common forms of RP as monitored by electroretinogram testing (61, 62). Because of heterogeneity of the RP mutations, it was important to correlate these findings with specific amino acid substitutions. This study has been extended to the specific T17M Rho mutation. Li et al. (63) found that vitamin A supplementation slows the rate of photoreceptor degeneration in mutant mice carrying this Rho mutation. In addition, supplementation with cis-retinoids also improves cell targeting of P23H Rho under tissue culture conditions (14, 15, 64). Thus, this simple but potentially effective therapy deserves more attention and further improvements.

Novel Properties of Pathological Mutant Opins—Opsin with a mutation that causes autosomal dominant RP may lead to an aberrant product that may, in turn, exhibit constitutive activ-
ity (65), have aberrant vectorial transportation (66), or misfold during biogenesis (14, 64, 67–69). The T4R mutation is characterized by opsin instability and may only manifest at a later stage of the disease when the supply of 11-cis-retinal is inhibited by the aging process or when the photoreceptors are exposed to intense light. We attribute these differences between the WT and T4R opsins to the positive charge at Arg4 resulting from the mutation and not the subsequent lack of glycosylation at Asn2, as bovine deglycosylated Rho in our assays showed very similar properties to WT Rho. However, lack of glycosylation at Asn15 may cause other cell biological problems, as a substitution of Ser for Asn (N15S) was identified in a patient with autosomal dominant RP (70, 71). In the case of human N15S and dog T4R mutations, the degeneration occurs within distinctive topographic regions (sectorial RP) (22, 70–72) that could be related to differences in light exposure. Detailed analysis of patients with the T4K mutation is needed to further support this hypothesis.

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