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ORIGINAL ARTICLE

ROLE OF RHODOPSIN PHOSPHORYLATION AT MULTIPLE SITES *IN VIVO*

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Abstract To study rhodopsin (Rho) phosphorylation at multiple sites *in vivo*, antibodies toward major Rho phosphorylation sites *in vivo*, 334Ser, 338Ser and 343Ser were prepared by immunization of authentic phosphorylated peptides to rabbit. Purified antibodies specifically recognized either the phosphorylated-334Ser, -338Ser or 343Ser site by ELISA. Immunofluorescence labeling by all these antibodies was exclusively light-dependent. However, their labeling patterns during light and dark adaptation were different from each other. Anti-P-Rho334 and P-Rho338 antibodies reacted with retinal photoreceptor outer segments (ROS) adapted by 650 and 5000 lux which caused Rho bleach at approximately 7 and 30% levels, respectively. However, in contrast, positive immunofluorescence labeling of ROS by anti-P-Rho343 was observed only in 5000 lux light-adapted rat retina. Similarly, strong, dim and negative stainings of ROS by anti-P-Rho334, P-Rho338, and P-Rho343 antibodies, respectively, were recognized in light adapted human retina by surgical illumination (approximately at 2000 lux) obtained from a patient with orbital malignant tumor. During dark adaptation of rat retina from light adaptation, immunoreactivities toward anti-P-Rho343 had diminished the fastest, followed by anti-P-Rho338 and then anti-P-Rho334. Our present results indicated that this newly developed method using specific antibodies toward different sites of phosphorylation is suitable for analysis of Rho phosphorylation and dephosphorylation *in vivo*.

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Key words: rhodopsin; phosphorylation; retinal degeneration; immunocytochemistry.

原　著

複数のリン酸化部位を有するロドプシンリン酸化の生体内における役割

大黒　浩　　大黒幾代　　中沢　満

抄録　脊椎動物視物質ロドプシンの３つのリン酸化部位（334Ser，338Serおよび343Ser）の生理的意義を検討するためにそれぞれのリン酸化部位を特異的に認識する抗体を作成する目的で３つのうち一つがリン酸化されたラットロドプシンペプチドを合成し，ウサギに免疫した．精製された抗体はELISA法でいずれか一つのペプチドのみを特異的に認識するものであった．これらの抗体を用いて免疫組織化学的検討を行ったところいずれの抗体も光退色した網膜視細胞を認識し，光退色していない網膜とは反応性が認められなかった．さらに 抗リン酸化ロドプシン334（P-Rho334）および抗リン酸化ロドプシン338（P-Rho338）抗体は650および5000luxの比較的弱い光条件および非常に明るい光条件（それぞれの光条件はロドプシンの７および30%を退色させる光量である）のいずれの条件で退色された視細胞を標識した．これに対して抗リン酸化ロドプシン343（P-Rho343）抗体は650luxの比較的弱い光条件では視細胞を標識せず，5000luxの非常に明るい光条件でのみ視細胞を標識した．これと同様に約2000luxの手術顕微鏡下で眼窩悪性腫瘍患者から摘出された眼球の網膜視細胞をそれぞれ抗P-Rho334，P-Rho338およびP-Rho343抗体によりかなり強く，比較的強くおよび非常に微弱に標識した．この結果はリン酸化された334Ser，338Serおよび343Serの脱リン酸化速度が順に速くなるという従来の生化学的検討の結果と一致する．従って本法のようなリン酸化部位を特異的に認識する抗体を用いた検討は*in vivo*のロドプシンリン酸化および脱リン酸化の検討に適していると考えられた．

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Introduction

In vertebrate rod outer segments (ROS), photoisomerization of rhodopsin (Rho) induced activation of thousands of heteroterimeric G-protein (Gt) which in turn activates cGMP phosphodiesterase (PDE), results in hyperpolarization of plasma membranes in response to the prompt decrease of cGMP concentrations in cytosol. This light-induced activation of this cascade reaction must be shut off and restored for next light stimulation. For this purpose, photoactivated Rho (Rho*) is regenerated by another cascade of reactions including 1) phosphorylation by rhodopsin kinase (RK), 2) binding of a regulatory protein called arrestin, 3) reduction of photolyzed chromophore (all-*trans* retinal), 4) dephosphorylation by protein phosphatase 2A, and 5) regeneration with 11-cis retinal. Among this cascade, it is known that phosphorylation and dephosphorylation of Rho is the most critical step in this regulation of the shut-off and restoration of the phototransduction cascade[1,2].

In terms of phosphorylation sites in Rho, *in vitro* studies revealed that light dependent phosphorylation occurs at multiple C-terminal Ser and Thr residues up to 7 Pi/Rho[3]. Pinpoint identification of the sites of Rho phosphorylation by direct sequencing or mass spectrometry identified that the initial sites of phosphorylation were 338Ser and 343Ser prior to multiple phosphorylation[4-6], and high stoichiometry of phosphorylation (4-7 Pi/Rho) was limited by the binding of arrestin to Rho*, and the reduction of the photolyzed chromophore, all-trans retinal, by retinol dehydrogenase[7]. In our previous study using living mice, we found that mono-phosphorylated at 334Ser, 338Ser or 343Ser was predominantly identified and only a little higher stoichiometry was detected upon illumination by using UV spectrometric quantification and mass spectrometry analysis[8]. Furthermore, we also found much faster kinetics of phosphorylation and dephosphorylation at 338Ser and 343Ser than at 334Ser during light and dark adaptation. Therefore, based upon these observations, we suggested that 338Ser/343Ser and 334Ser are involved in different roles in the visual transduction. In a subsequent *in vivo* study, Hurley and his associates also reported predominant mono-phosphorylation of Rho at 343Ser, 338Ser and 334Ser as well as multiple phosphorylation as minor components in similar analysis using mass spectrometry but employing a much faster quenching system[9,10]. However, there were some differences between their and our studies; e. g., preferable initial phosphorylation site, 343Ser vs 338 Ser, and their kinetics of phosphorylation and dephosphorylation.

In order to elucidate which sites of Rho phosphorylation are importantly involved in visual transduction *in vivo*, we developed a new method using antibodies for identifying Rho phosphorylation at specific sites[11]. Preliminarily, we raised specific antibodies toward major Rho phosphorylation sites *in vivo*, 334Ser or 338Ser by immunization of authentic phosphorylated peptides in rabbit. In immunofluorescence labeling, both antibodies recognized photoreceptor outer segments in light-adapted rat retinas. During dark adaptation, immunoreactivities toward phosphorylated-338Ser and -334Ser sites were diminished within several hours (0.2-4 hr). This data corresponds well with previous observations obtained by biochemical assays using [^{32}P] tracer and mass spectrometry analysis[8]. Thus, we suggested that this newly developed method using specific antibodies toward phosphorylated Rho at specific sites is a suitable for studying

phosphorylation and dephosphorylation of Rho *in vivo*. Therefore, in the present study, to gain additional insight into the role of Rho phosphorylation at multiple sites *in vivo*, we prepared specific antibody toward another major Rho phosphorylation site 343Ser in addition to 338Ser and 343Ser and immunohistochemical analysis was performed.

Material and Methods

Unless otherwise stated, all procedures were performed at 4℃ or on ice using ice-cold solutions. Sprague Dawley (SD) rats (Crea, Tokyo, Japan) reared in cyclic light conditions (650 lux, 12 hours on / 12 hours off) were used. Authentic phosphorylated peptides, P-Rho334-peptide (DEApSATASK), P-Rho338-peptide (CEASATApSKT), P-Rho343-peptide (CDEASATAAKTET(pS)QVAPA) and non-phosphorylated peptides(DEASATAAKTE TSQVAPA) were purified by a C18 HPLC column (Cosmosil $5C_{18}$-P300, Nacalai Tesque, Japan) using an acetonitrile gradient from 0-80% in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min (purities>98%). Rat ROS and urea-washed ROS membranes were prepared using 200 freshly dissected retinas from dark adapted SD rats as described previously[12]. Rho concentrations were determined in 10 mM hepes buffer, pH 7.5 containing 10 mM chaps from the difference in absorbance at 500 nm before and after bleaching in the presence of NH_2OH by assuming ε_{500} to be 41000 and the molecular weight to be 38000. A light adapted intact human eye was surgically obtained from a 60 year-old male patient with orbital malignant tumor under surgical illumination (approximately 2000 lux) and thereafter fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) followed by immunohistochemical analysis described below. Informed consent was obtained.

Preparation of specific antibodies toward phosphorylated Rho at 334Ser, 338Ser or 343Ser.

Specific antisera toward phosphorylated Rho at 334Ser (anti-P-Rho334), 338Ser (anti-P-Rho338) or 343Ser (anti-P-Rho343) were obtained by immunization of phosphorylated authentic peptides P-Rho334 peptide, P-Rho338 peptide, or P-Rho343 peptide chemically conjugated with bovine thyroglobulin and complete adjuvant to rabbit. Antisera were each further purified into IgG[13], and 0.1 mg of them was each incubated with urea-washed ROS (20 mg of Rho) at room temperature for 2 h. Then the mixture was ultracentrifuged at 100,000 x g for 1 h. The resultant supernatant was used as specific antibody toward phosphorylated Rho at 334Ser, 338Ser or 343Ser.

Preparation of phosphorylated photolyzed Rho (P-Rho*)

After overnight dark adaptation, fresh ROS (5 mg of Rho) obtained from 6 to 8-week old SD rats 12) was dissolved in 5 ml of buffer A (100 mM Na-phosphate buffer, pH 7.2, 5 mM $MgCl_2$, 100 mM KCl) containing 1 mM ATP and incubated at 30℃ for 10 minutes under a 150-W lamp from a distance of 20 cm. The reaction was terminated by the addition of buffer B (200 mM Na-phosphate buffer, pH 7.2 containing 5 mM adenosine, 100 mM KF and 200 mM EDTA). P-Rho* pellet was obtained by centrifugation.

Immunofluorescence microscopy

Under deep ether anesthesia, rats kept under different illumination conditions were perfused with 300 ml of 4% paraformaldehyde in PBS. Fixed rat and human retinas as above were dissected out and embedded in paraffin. After the deparaffinization, retinal sections vertically through the optic disc at 4

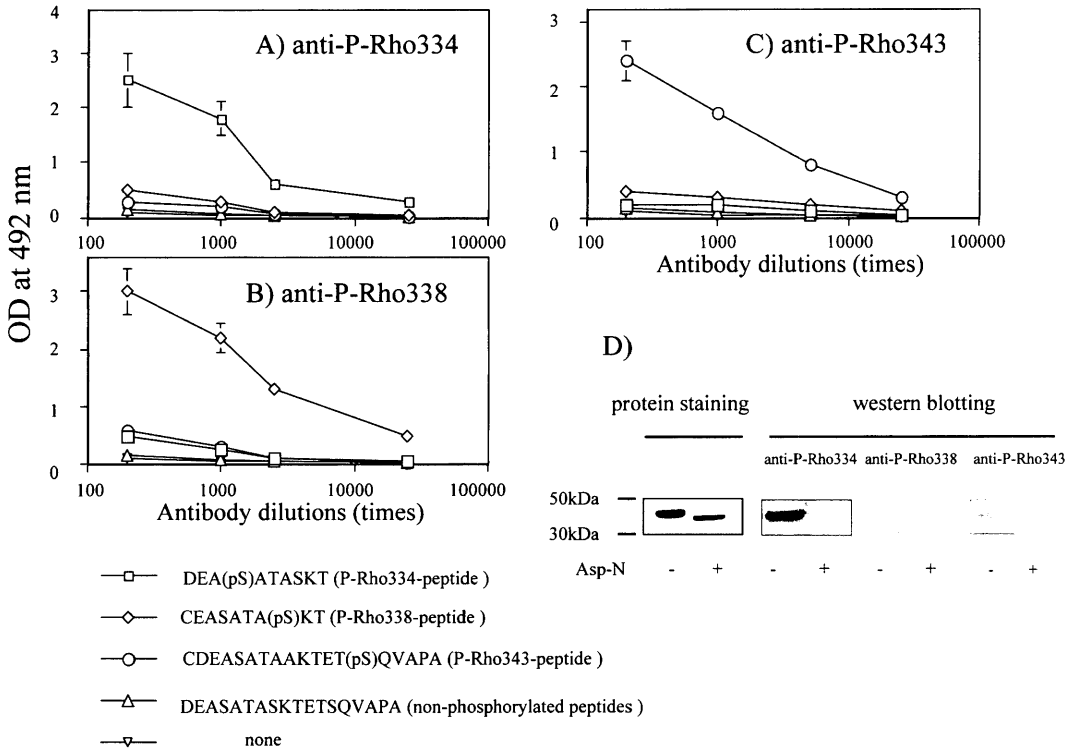

Figure 1 Characterization of anti-P-Rho334, anti-P-Rho338 and anti-P-Rho343 IgG.
Affinity purified anti-P-Rho334, anti-P-Rho338 or anti-P-Rho343 IgG were subjected to ELISA (A, B and C) using authentic peptides. Western blot analysis was performed using endoproteinase Asp-N treated or non-treated P-Rho* (D). ELISA was performed in triplicate. Data are expressed as the mean ± SD.

μm thickness were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h and then incubated successively with anti-P-Rho antibodies (1:500) and FITC-conjugated anti-rabbit IgG (Cappel, Durham, NC) for 1 h each at room temperature.

ELISA and western blot analysis

Authentic peptides (50 μg/ml) in PBS were coated onto a 96-well microtiter plate (Dynatech. CO.) overnight at 4℃. After blocking with 1% BSA for 1h at room temperature, the wells were incubated successively with anti-peptides antibodies at graded dilutions (0-25000 times) and HRP-labeled anti-rabbit IgG (1:3000 dilutions, Cappel, Durham, NC) for 1h at 37℃. The wells were extensively washed with 0.035% Tween 20 in PBS and antigen-antibody binding was visualized with o-phenylenediamine as a substrate. The OD was measured at 492 nm, primary and secondary antibodies were probed at 1 : 2000 dilutions and specific antibody/antigen binding was visualized by ECL (Amersham) [13]. Approximately 10 μg of P-Rho* treated or untreated by Asp-N (enzyme to substrate ratio at 1 : 1000) for overnight at room temperature was subjected to western blot analysis. Electrotransferred blots were probed primary and secondary antibodies at 1 : 2000 dilutions and specific antibody/antigen binding was visualized by ECL (Amersham) [13].

Results

As shown in Figure 1 (A-C), anti-P-Rho334, anti-P-Rho338 or anti-P-Rho343 IgG exclusively recognized P-Rho334, P-Rho338 or P-Rho343 peptide, respectively, with no immunoreactivities toward

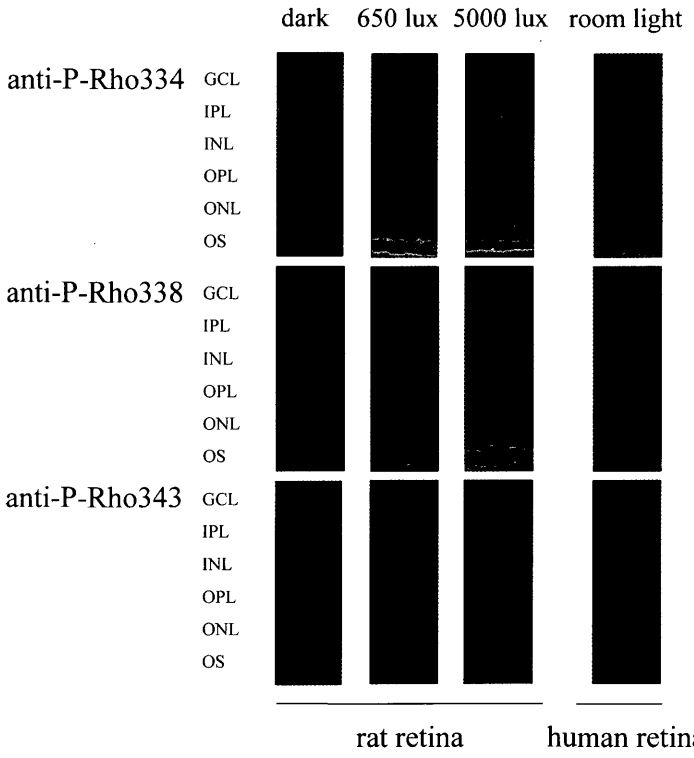

Figure 2 Immunofluorescence labeling of anti-P-Rho334, anti-P-Rho338 or anti-P-Rho343 IgG in light- or dark-adapted rat retinas and light-adapted human retina.
Dark or light-adapted rat retinas by 650 lux or 5000 lux illumination and light-adapted human retina by surgical halogen lamp (2000 lux) were subjected to immunofluorescence labeling by anti-P-Rho antibodies. GCL, ganglion cell layer, IPL, inner plexiform layer, INL, inner nuclear layer, OPL, outer plexiform layer, ONL, outer nuclear layer, OS, outer segment. Scale bar=50 μm.

other peptides. Western blot analysis demonstrated that all these anti-P-Rho IgGs reacted with rat P-Rho, but not with endoproteinase Asp-N treated P-Rho which is missing the C-terminal 19 amino acids, 330DDEASATASKTETSQVAPA, containing all possible phosphorylation sites (Figure 1D).

In immunofluorescence labeling, no immunoreactivities were recognized in dark adapted rat retinas by all these antibodies and specific labeling within ROS was detected in light adapted rat and human retinas (Figure 2). However, the immunoreactivities among antibodies were different under several illumination conditions. In rat retinas, anti-P-Rho334 and anti-P-Rho338 IgG recognized both mild (650 lux, approximately 7% Rho bleach) and high (5000 lux, approximately 30% Rho bleach) bleached retinas. However, in contrast, anti-P-Rho343 IgG reacted with only high bleached retina (5000 lux), but not with dark and mild bleached (650 lux) retinas. In human retinas, which were obtained under surgical illumination (approximately 2000 lux), positive imunoreactivities were recognized by anti-P-Rho334 and anti-P-Rho338, but insignificant labeling by P-Rho343 antibodies. Furthermore their immunoreactivities were different; anti-P-Rho334 and anti-P-Rho338 antibodies immunoreacted with top half and tip of ROS, respectively. Therefore, these data indicated different kinetics and spartial phosphorylation of Rho* at multiple C-terminal Ser sites *in vivo* as indicated by previous studies[8].

To further elucidate physiological significance of Rho phosphorylation at different sites *in vivo*, their dephosphorylation

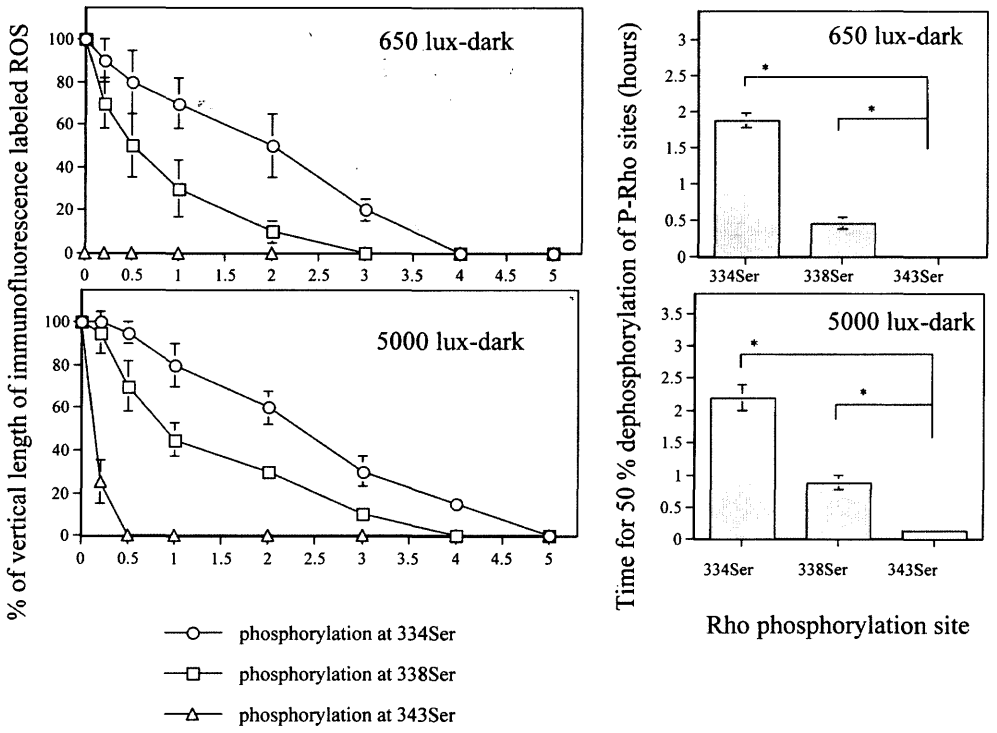

Figure 3 Kinetics of dephosphorylation in phosphorylated 334Ser, 338Ser and 343Ser sites in SD rat retina.
Left panels: Five-week-old SD (n=21 rats) kept under illumination condition (650 lux or 5000 lux) for 1 hr were incubated under dark condition. At eight different time points (0, 0.2, 0.5, 1, 2, 3, 4 and 5 hrs), two consecutive sections from three rats (6 eyeballs) in each category were subjected to HE stainings and immunofluorescence labeling by anti-P-Rho antibodies. Photographs of the sections were taken. Vertical length of photoreceptor outer segment layers of HE staining section and that of fluorescence labeling were measured at temporal points 1.0 mm apart from optic disc from 6 different points from 6 different eyeballs and their ratios were plotted. **Right panels:** Times requiring for 50% dephosphorylation at these three different Rho phosphorylation sites under different illumination conditions were plotted. Data are expressed as the mean ± SD. *$p<0.01$ (MannWhitney).

following dark adaptation was studied by a recently developed method to measure vertical lengths of immunofluorescence labeled ROS during the dark adaptation[11]. During the dephosphorylation of both sites, this method showed that tips of ROS were slower than bases, as similarly observed in our previous study using mice[8]. As shown in Figure 3 left panels, kinetics of dephosphorylation of phosphorylated 334Ser, 338Ser and 343Ser sites of SD rats were compared during exposure to different bleach conditions prior to the dark adaptation. In the dark adaptation from mild bleach condition (650 lux), dephosphorylation of 334Ser and 338Ser sites went to completion within 3 or 4 h, respectively, and no phosphorylation at 343Ser was recognized under this conditions. While dephosphorylation of 334Ser and 338Ser sites under dark adaptation from high bleach (5000 lux) was slower than under mild bleach conditions, prompt dephosphorylation of 343Ser was apparent within 0.5 h. As shown in right panels, times for requiring 50% dephosphorylation at these three phosphorylation sites during dark adaptation were significantly different with each other from mild (650 lux) and high (5000 lux) bleach conditions.

Discussion

In the present study, *in vivo* Rho

phosphorylation and dephosphorylation were studied employing a new method utilizing specific antibodies toward phosphorylation sites of Rho at 334Ser, 338Ser and 343Ser sites, and the following findings were observed; 1) Raised antibodies specifically recognized a corresponding peptide within one of these sites of phosphorylation and no cross reactions with other peptides. 2) Positive immunofluorescence labeling by these antibodies was observed within ROS in only light-adapted but not in dark-adapted SD rat and human retinas. 3) Their positivities exclusively depended on their bleaching levels. That is, anti-P-Rho334 and anti-P-Rho338 immunoreacted with bleached retina at both low (650 lux, approximately 7% Rho bleach) and high (5000 lux, approximately 30% Rho bleach) levels of bleaching. In contrast, positive immunolabeling by anti-P-Rho343 was only recognized in higher bleached retina (5000 lux). 4) During dark adaptation, decay of the immunolabelings by anti-P-Rho343 was the fastest, followed by anti-P-Rho338 and then anti-P-Rho334. Furthermore, it was notably shown that time requiring for 50% dephosphorylation at these three phosphorylation sites from different bleaching levels were exclusively different among these phosphorylation sites and illumination conditions. Therefore, different kinetics of Rho phosphorylation and dephosphorylation at specific sites were pivotal for understanding molecular mechanisms of light and dark adaptation of photoreceptor cells under different illuminating conditions, and time requiring for 50% Rho dephosphorylation at specific sites may be an useful indicator for evaluating Rho phosphorylation and dephosphorylation kinetics *in vivo*.

Rho phosphorylation and dephosphorylation has been extensively investigated by several methods including biochemical assays using radioactive phosphate, ion-exchange column chromatography method, electrophoretical method and mass-spectrometry-based method (reviewed by ref 2). In every method, a consistent observation is that Rho phosphorylation and dephosphorylation are exclusively light-dependent. However, kinetics, stoichiometry and sites of Rho phosphorylation were different depending on which methods were employed. In terms of sites of phosphorylation of Rho, almost all studies consistently reported main sites of Rho phosphorylation at 334Ser, 338Ser and 343Ser *in vitro* and *in vivo*[4-10]. However, preferential sites were different in assay systems based on bleaching and time scale of quenching. In fact, our previous study identified 338Ser and 334Ser as primary sites of phosphorylation in living mice[8]. Recent studies using mass-spectrometry based analysis in conjunction with more rapid quenching system revealed that phosphorylation at 343Ser becomes significant in addition to the above two sites[9,10]. This difference is most likely caused by much faster kinetics of dephosphorylation at 343Ser than that at 338Ser and 334Ser identified by previous and present studies[8,9].

This newly developed method for detecting specific phosphorylation site by antibody seems to be quite useful for studying Rho phosphorylation and dephosphorylation *in vivo*. Similarly Adamus et al. recently described that the kinetics of phosphorylation and dephosphorylation were consistently slower at 334Ser than 338Ser in mice, based on a similar method using specific antibody against these phosphorylation sites. In that study, they suggested that the difference in the kinetics was caused by involvement of a different kinase-based phosphorylation

system; 338Ser by Rho kinase and 334Ser by protein kinase C[14]. In our previous study, this methodology also identified extremely prolonged kinetics of dephosphorylation in Royal College of Surgeons (RCS) rat, an animal model for human retinitis pigmentosa (RP)[11]. Thus, we suggested that prolonged survival of phosphorylated forms of Rho may contribute persistent misregulation of phototransduction processes and may explain the reason why PR patients notice night blindness since their early disease stages. Furthermore, this methodology will be applicable to identify the sites and study the kinetics of phosphorylation of cone pigments, which are still unknown.

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