High levels of rhodopsin phosphorylation in missense mutations of C-terminal region of rhodopsin

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Abstract Rhodopsin phosphorylation was investigated using synthetic C-terminal peptides from rhodopsin. The peptides were phosphorylated by expressed rhodopsin kinase (RK) in the presence of a photolyzed truncated rhodopsin at the C-terminus. No peptide phosphorylation was detected under dark or in conditions in which RK was inactive. However, the phosphorylation rate was significantly higher in the following three peptides: (345M Rho, 330DDEASTTVSKTETSQMAPA; 347S Rho, 330DDEASTTVSKTETSQVASA; and 347L Rho: 330DDEASTTVSKTETSQVALA) taken from missense mutations of rhodopsin found in patients with autosomal retinitis pigmentosa (ADRP) as compared with that from wild-type rhodopsin (330DDEASTTVSKTETSQVAPA). Distribution of the phosphorylation showed a similar ratio among three serines (334, 338 and 343) in 347L Rho mutation to wild type. However, 345M Rho and 347S Rho peptides showed higher phosphorylation at Ser^{334}. The data obtained suggests that an abnormally high rate of phosphorylation in missense mutations around the rhodopsin C-terminus may change the position of phosphorylation and inactivation process of the visual transduction.

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Key words: Rhodopsin phosphorylation; Rhodopsin; Rhodopsin kinase; Retinitis pigmentosa

1. Introduction

Retinitis pigmentosa (RP) is a group of an inherited disease of progressive retina degeneration with characteristic features including nyctalopia, ring scotoma, and bone spicule pigmentation of the retina. A major cause of the autosomal dominant form of RP (ADRP) is mutation of the rhodopsin gene [1,2]. More than 60 different rhodopsin mutations distributed all regions of rhodopsin, intradiscal, transmembrane, cytoplasmic loop and C-terminal domains have been described [2].

Rhodopsin is the most abundant membrane-spanning protein in the rod outer segments. Following absorption of a photon, rhodopsin isomerizes into an active photobleaching intermediate (metarhodopsin II), which initiates a G-protein (Gt) mediated amplifying cascade of reactions to generate visually evoked signals in photoreceptor cells [3]. This cascade is shut down by phosphorylation of photolyzed rhodopsin by rhodopsin kinase (RK) resulting in blockage of further interaction of metarhodopsin II with Gt by binding with arrestin [3]. In vitro and in vivo studies revealed that light-dependent phosphorylation of rhodopsin occurs at one of three serine (Ser^{334}, Ser^{338} and Ser^{343}) and one threonine (Thr^{336}) residues at the C-terminal segment [4–8]. Within the C-terminal cytoplasmic domain, the four terminal amino acids, Val^{345}-Ala-Pro-Ala, are highly conserved in mammals, and Val^{345} and Pro^{347} are the most common sites of mutations causing ADRP [2]. The disease phenotype can be mild or severe. Studies by site-directed mutagenesis showed that these missense mutants possess wild-type biochemical properties including gene product synthesis, transport and immunogenic properties [9,10]. The molecular pathophysiology of retinal degeneration in ADRP with these mutations is still unknown. However, the above information led us to speculate that the missense mutations in this region may effect rhodopsin phosphorylation.

Here, to test this hypothesis, we studied phosphorylation of rhodopsin with missense mutations near the phosphorylation sites using a peptide phosphorylation system and found significantly increased levels of rhodopsin phosphorylation in these missense mutations.

2. Materials and methods

2.1. Materials

A bovine rhodopsin 19-amino-acid C-terminal peptide (330DDEASTTVSKTETSQVAPA) and three peptides with missense mutations (330DDEASTTVSKTETSQMAPA, 330DDEASTTVSKTETSQVASA, and 330DDEASTTVSKTETSQVALA) were purchased from Quality Controlled Biochemicals (MA). The sequences were verified by amino-acid composition and Edman sequence analysis. Rhodopsin truncated at Gly^{329} (D-Rho) was prepared by proteolysis of urea-washed rhodopsin by endoproteinase Asp-N, and recombinant RK was expressed in insect cells and purified as described previously [11]. All other reagents used were analytical grade.

2.2. Phosphorylation of rhodopsin C-terminal peptides

Synthetic rhodopsin C-terminal 19-amino-acid peptides (66 μM) were phosphorylated in a total of 150 μl of 50 mM BTP (bis-tris-propane) buffer containing 5 mM $MgCl_2$ in the presence of D-Rho (50 μM), recombinant RK (300 pmole of phosphate transfer/min/kinase activity) and 0.167 mM [^{32}P]ATP (300 cpm/nmol) at 30°C for 30 min under a 150-W lamp from a distance of 30 cm. The reaction was terminated by adding 10% acetic acid. The mixture was passed through a DEAE cellulose column (5×10 mm) which had been equilibrated with 5% acetic acid. A large excess of ATP bound to the column. Phosphorylated and unphosphorylated rhodopsin C-terminal peptides unbound to the column were directly injected to a HPLC reverse phase column (2.1×250 mm, C18 column, Vydac 218TP52) and purified employing a linear gradient of acetonitrile from 0% to 24% and from 0.08% to 0.1% trifluoroacetic acid during 40 min at a flow rate of 0.3 ml/min. Fractions (0.3 ml each) were collected and the radioactivities were counted by Cerenkov's method.

2.3. Determination of the site of phosphorylation

The phosphorylation sites of rhodopsin C-terminal peptides were estimated from the radioactive ^{32}P-elution profiles of their subdigestion by trypsin as described by Ohguro et al. [7]. Briefly, C-terminal phosphorylated peptides were each treated with 1 μg of TPCK-trypsin (Worthington) at 30°C for 1 h and directly injected to a C18 reverse phase HPLC column (2.1×250 mm, Vydac218TP52). Peptides phos-

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phorylated at different sites were separated by elution with a linear gradient from 0% to 24% acetonitrile and from 0.08% to 0.1% trifluoroacetic acid for 60 min at a flow rate of 0.3 ml/min. Fractions (0.3 ml each) were collected and the radioactivities were counted by Cerenkov's method.

3. Results and discussion

It was found that authentic peptides corresponding to the C-terminal region of rhodopsin can be phosphorylated by RK in the presence of photobleaching intermediates of rhodopsin, or the truncated form of rhodopsin at the C-terminus [12,13]. Akhtar and his colleague [12,14,15] have shown that the kinetics and distribution of phosphorylation of the peptide were identical with that of native receptors although the rate of phosphorylation is relatively low (less than 10%). By utilizing this methodology, we have studied phosphorylation of rhodopsin with missense mutations at the C-terminus found in ADRP in order to elucidate its molecular pathophysiology. For the selection of the authentic peptide, the following previous observations were taken into account: (1) bovine rhodopsin is the best studied species; (2) serine residues at 334, 338 or 343 are the initial sites of phosphorylation in vitro and in vivo studies [4–8]; (3) 9–12 amino acids from the C-terminal region were preferable substrates for RK, but the stoichiometry and the distribution were changeable by the position (one residue on the amino side and five on the acyl side were at least required for suitable phosphorylation) [12,14,15]; (4) a C-terminal 19-amino-acid peptide obtained by endoproteinase Asp N was extensively characterized [4–8,11,13]. From these observation, we designed a reconstituted experiment using a C-terminal 19-amino-acid peptide from bovine rhodopsin, 330DDEASTTVSKTETSQVAPA, and a truncated form of rhodopsin at Gly^{329} (D-Rho) eliminated the 19 amino acids,

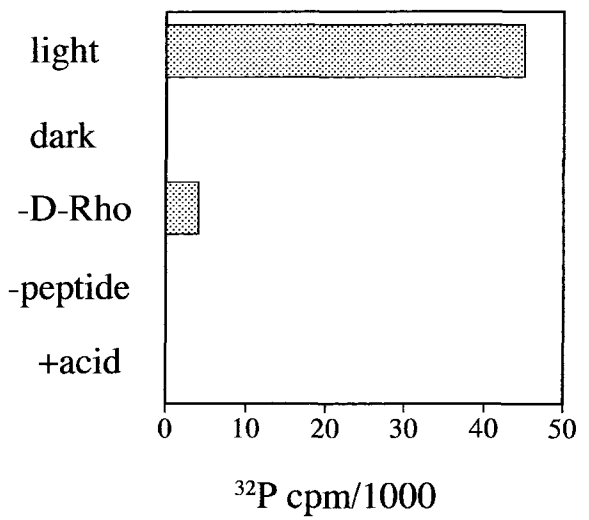


Fig. 1. Phosphorylation of rhodopsin C-terminal peptide catalyzed by rhodopsin kinase. Phosphorylation of rhodopsin C-terminal peptide, 330DDEASTTVSKTETSQVAPA, was catalyzed by expressed RK in the presence of photolyzed D-Rho and [^{32}P]ATP as described in Section 2. As a control, experiments were performed under several conditions in the dark, without peptide, or conditions where endogenous and exogenous RK were inactivated by adding acid. Phosphorylated and unphosphorylated peptides were purified by HPLC column and their radioactivities were counted. Experiments were performed in duplicate.

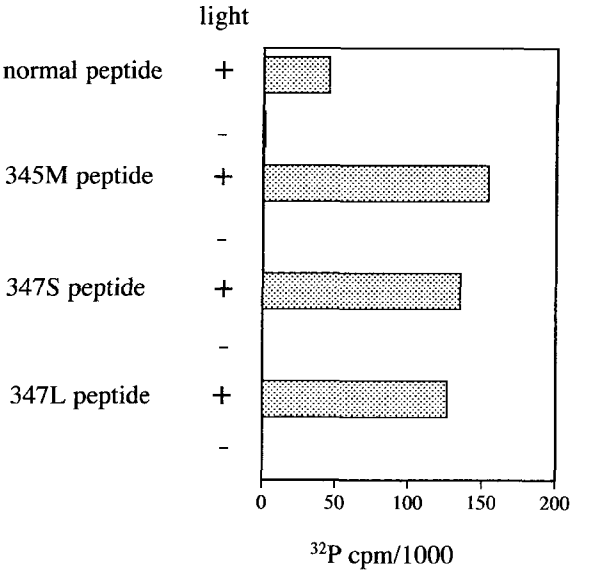


Fig. 2. Phosphorylation of C-terminal peptides of rhodopsin with missense mutations in ADRP by RK. Three peptides (345M Rho, 330DDEASTTVSKTETSQMAPA; 347S Rho, 330DDEASTTVSKTETSQVASA; and 347L Rho, 330DDEASTTVSKTETSQVALA) from missense mutations observed in patients with ADRP were phosphorylated by RK in the presence of D-Rho under dark or light conditions. Phosphorylated and unphosphorylated peptides were purified by HPLC column and their radioactivities were counted. Experiments were performed in duplicate.

and expressed RK. As shown in Fig. 1, the peptide was remarkably phosphorylated by the expressed RK under white light illumination in the presence of D-Rho, while no phosphorylation was detected in conditions without the peptide or D-Rho, or under dark conditions. The peptide was also unphosphorylated when endogenous and exogenous RK were inactivated by adding acid to the mixture. Thus, phosphorylation of the rhodopsin peptide occurred in a light and RK dependent manner like native forms, and is suitable for study further using mutant peptides.

Three peptides (345M Rho, 330DDEASTTVSKTETSQMAPA; 347S Rho, 330DDEASTTVSKTETSQVASA; and 347L Rho, 330DDEASTTVSKTETSQVALA) from missense mutations frequently observed in patients with ADRP [2] were synthesized and examined for their phosphorylation properties. As shown in Fig. 2, all peptides were phosphorylated by RK in a light-dependent manner, but the rates of phosphorylation of these mutant peptides were twice to three times higher than that in the wild-type peptide. The distribution of mono-phosphorylation at Ser^{334}, Ser^{338} or Ser^{343} in 347L Rho peptide was almost identical to that in the normal peptide, while 347S Rho and 345M Rho peptides showed significantly higher rates of phosphorylation at Ser^{334} site (Fig. 3).

In our previous study using mice, we found different kinetics of phosphorylation and dephosphorylation among Ser^{334}, Ser^{338} and Ser^{343} sites [8], and suggested that rhodopsin phosphorylation at specific sites may be involved in the different inactivation processes, such as quenching and adaptation. In vitro, the distribution of rhodopsin phosphorylation among the three serines were not affected by different bleaching states of rhodopsin (meta I, meta II, meta III), removal of palmitiol groups at $Cys^{322}Cys^{323}$ that anchor the C-terminal domain to disc membrane, nor by other factors (arrestin, ret-

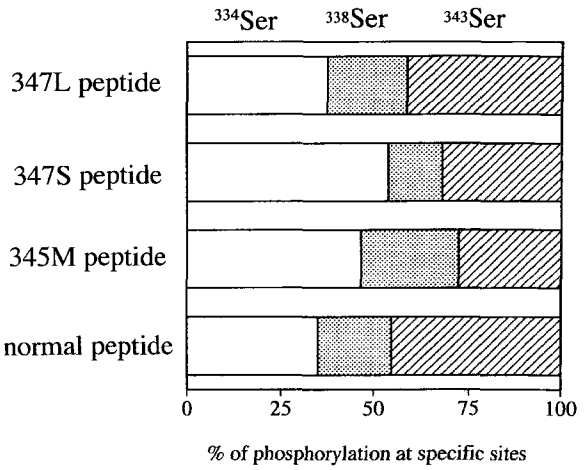


Fig. 3. Distribution of phosphorylation on the C-terminal peptides from normal and mutant rhodopsin. Percents of mono-phosphorylated rhodopsin at different sites, Ser^{334} (open), Ser^{338} (dot), or Ser^{343} (stripe) were determined by radioactive ^{32}P-profiles of the trypsin digests of the C-terminal peptides as described in Section 2. Experiments were performed in duplicate.

inol dehydrogenase, Gt and recoverin). However, truncation of the C-terminal four to nine amino acids of rhodopsin by proteolytic enzymes led to different stoichiometry of phosphorylation among the three serines, and unique initial phosphorylation at Thr^{336} which has never been found before [11]. Taken together with the current data, it was concluded that the C-terminal tail of rhodopsin is a key region conducive to the kinetics and distribution of phosphorylation.

The molecular pathophysiology of ADRP with missense mutations has not been elucidated yet. Mutagenesis studies have hypothesized that prolonged activation of the rod caused by constitutive activation of Gt could lead to cell death by way of unknown events [16–18]. A prolonged desensitization caused by an absence or decrease of rhodopsin phosphorylation is a critical factor of retinal degeneration as shown by the following observations: (1) Absence of rhodopsin phosphorylation was found in some rhodopsin mutants [19]; (2) Transgenic mice carrying rhodopsin C-terminal truncation at Gly^{329} [20] or Glu^{344} [21] showed prolonged responses and retinal degeneration. However, in contrast, missense mutations within the rhodopsin C-terminus most frequently found in ADRP showed an increase of phosphorylation as described above. This process may involve accelerated desensitization producing a change of status of visual transduction pathways also resulting in retinal degeneration. However, our understanding of the above process and how it leads to cell death is at present speculative and consequently further study is required.

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