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Opsins with mutations at the site of chromophore attachment constitutively activate transducin but are not phosphorylated by rhodopsin kinase

(visual pigment/constitutive activity)

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ABSTRACT **More than 70 mutations in the gene encoding the visual pigment rhodopsin have been identified in patients with autosomal dominant retinitis pigmentosa. Most of these mutations are thought to interfere with proper folding of the membrane protein. However, families with a severe phenotype of retinitis pigmentosa have been identified and shown to carry a mutation at the site of chromophore attachment, Lys-296. This mutation disrupts the inactive conformation of opsin and results in a constitutively active protein that can activate the rod-specific GTP-binding protein, transducin, in the absence of light and in the absence of the chromophore 11-*cis*-retinal. It has been suggested that this mutant opsin molecule may cause rod degeneration by depletion of the components used to inactivate rhodopsin, such as rhodopsin kinase. In this work we test this idea by determining whether two constitutively active opsin mutants are phosphorylated by rhodopsin kinase. We found that opsin mutants where Lys-296 is replaced either by Glu (K296E) or by Gly (K296G) are not substrates of rhodopsin kinase in the absence of chromophore. However, when K296G is regenerated with a Schiff base complex of 11-*cis*-retinal and *n*-propylamine and exposed to illumination, phosphorylation of opsin occurs. These experiments suggest that in the rod photoreceptors of patients with retinitis pigmentosa carrying a mutation at Lys-296, there is persistent activation of the GTP-binding protein-mediated cascade. This may result in a situation that mimics long-term exposure to continuous illumination and results in the degeneration of photoreceptors.**

The visual pigment rhodopsin is a member of a large family of receptors that mediate their effects through GTP-binding (G) proteins (1–8). Rhodopsin contains an 11-*cis*-retinal chromophore that is covalently attached via a protonated Schiff base linkage to the apoprotein, opsin (9–11). The effect of light is to isomerize the chromophore to the all-*trans* configuration resulting in photoactivated rhodopsin (R*) (12). In the rod photoreceptor, R* binds and activates the G protein, transducin (G_t), initiating a biochemical cascade that results in a transient decrease in intracellular cGMP. A specific conformation of R* identified as the spectral intermediate, metarhodopsin II (MII), activates G_t. Two other proteins, arrestin and rhodopsin kinase (RK), involved in the deactivation of R*, have also been shown to bind to MII (13, 14). MII is unusual in that the Schiff base linkage to the isomerized all-*trans*-retinal is deprotonated and the carboxylic acid of Glu-113 appears to become protonated (15–17).

Recent mutagenesis studies have demonstrated that mutations of Glu-113, Glu-134, Lys-296, Gly-90, or Ala-292 in opsin result in constitutive activation of the protein as assayed by its ability to activate G_t in the absence of chromophore and light (18–20, 37). The mutations at position 296 are particularly interesting because one of them, K296E, is associated with a severe phenotype of autosomal dominant retinitis pigmentosa (21). It has been hypothesized that the persistent activation of the signaling pathway may cause retinitis pigmentosa by depleting the photoreceptor of RK and arrestin (18). To test this hypothesis, we assayed the ability of RK to phosphorylate K296E and K296G. We found that these two mutant apoproteins were not substrates for RK, suggesting that K296E persistently activates G_t. This result also suggests the possibility of at least two active conformations of R*, one that can bind and activate G_t and another that can bind and serve as a substrate for RK.

MATERIALS AND METHODS

Materials. All reagents except where indicated were purchased from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was purchased from DuPont/New England Nuclear. Dodecyl β-D-maltoside was obtained from Calbiochem. Recombinant N-Glycanase was obtained from Genzyme. Fresh bovine retina were obtained from a local slaughterhouse. 11-*cis*-Retinal was the generous gift of the National Eye Institute. Rhodopsin antibodies were the generous gift of R. S. Molday (1D4; University of British Columbia, Vancouver) and Paul A. Hargrave (B6-30; University of Florida, Gainesville). The epitope recognized by 1D4 contains the last 8 amino acids in the C terminus, and the epitope recognized by B6-30 contains amino acids 3–14 in the N terminus. The Schiff base complex of 11-*cis*-retinal with *n*-propylamine (nPrSB) was prepared as described (22).

Mutagenesis of the Rhodopsin Gene and Expression of Mutants. All procedures for DNA manipulation, mutation of the bovine rhodopsin gene, DNA sequence analysis, transfection, expression of the rhodopsin gene in COS cells, and preparation of membranes from transfected COS cells were performed as described (18, 19, 23, 24).

Purification of RK. RK was purified and its activity was determined as described in Buczyłko *et al.* (25).

Assay for RK Activity Using Opsin and Opsin Mutants Expressed in COS Cells. Wild-type rhodopsin and rhodopsin mutants expressed in COS cell membranes were assayed for their ability to act as a substrate for purified RK. COS cell membranes were assayed in the absence of chromophore or were regenerated with chromophore: native opsin with 11-*cis*-retinal or the mutant K296G with nPrSB. Membranes were incubated with 200 μM chromophore for 1 h in the dark at room temperature prior to use. The concentrations of all

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Abbreviations: G protein, GTP-binding protein; R*, photoactivated rhodopsin; G_t, transducin; MII, metarhodopsin II, nPrSB, Schiff base complex of 11-*cis*-retinal with *n*-propylamine; RK, rhodopsin kinase.

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opsins were ≈50 nM, as estimated by immunoblot analysis. Membranes were then centrifuged and resuspended in 50 mM 1,3-bis[tris(hydroxylmethyl)methylamino]propane (BTP) (pH 7.5) containing 1 mM $MgCl_2$. COS cell membranes were concentrated 10-fold by this procedure. Membranes (50 μl) were added in the dark to a reaction mixture containing 5 μl of [γ-^{32}P]ATP (1.6 mM, 1200 cpm/pmol; yielding a final concentration of 75 μM ATP) and 1 μg of purified RK. This mixture was incubated at 20°C in the dark or under illumination from a 150-W lamp. At 0, 2, 6, 10, and 20 min, 20 μl was added to 5 μl of 25 mM EDTA/100 mM dodecyl β-D-maltoside in 50 mM BTP (pH 7.5). This suspension was deglycosylated using the procedure described below or immediately analyzed by SDS/PAGE. Radiolabeled protein bands were visualized using autoradiography and the amount of radioactivity incorporated into each band was determined as described (25).

Deglycosylation of Rhodopsin and Rhodopsin Mutants Expressed in COS Cells. The glycosylation of rhodopsin expressed in COS cells is variable. When opsin was examined by SDS/PAGE and immunoblot analysis, multiple bands were visualized resulting in a smeared appearance. To improve our ability to visualize rhodopsin phosphorylation in the assay described above, a procedure was developed to cleave the variable length sugars that modify COS cell rhodopsin. Three units of N-Glycanase (3 units/mg of protein, where 1 unit is the amount of enzyme required to catalyze the release of Asn-linked oligosaccharide from 1 nmol of [^{3}H]dansyl-fetuin glycopeptide per min at 37°C and pH 8.6) was added to the quenched samples from the phosphorylation reaction and the sample was incubated in the dark at 20°C for 3 h.

Identification of Phosphorylation Sites in Rhodopsin and the Opsin Mutant K296G. COS cell membranes containing regenerated wild-type opsin or the regenerated opsin mutant K296G were phosphorylated by RK as described above. ^{32}P-phosphorylated COS cell membranes containing 2–4 μg of rhodopsin or the mutant K296G were mixed with 1 mg of nonradioactive phosphorylated rhodopsin, which was used as a carrier protein. The C-terminal peptide, ^{330}DDEASTTVSKTETSQVAPA, containing all RK phosphorylation sites was isolated by proteolysis with endoproteinase Asp-N (Boehringer Mannheim). The enzyme to rhodopsin ratio was 1:7000 (wt/wt) and the digestion was performed in 10 mM Hepes (pH 7.5) for 12 h at 18°C (26). The soluble C-terminal peptide was separated from membranes by centrifugation at 200,000 × *g* for 20 min and purified using a C_{18} HPLC column (2.1 × 250 mm, Vydac 218T52) employing a linear gradient from 0 to 52% (vol/vol) acetonitrile in 0.08–0.1% trifluoroacetic acid. Peptides were detected at 220 nm (27). For mapping the phosphorylation sites, the peptide product of the Asp-N cleavage was subjected to a combination of further digestions with thermolysin and trypsin and purification by HPLC as described by Ohguro *et al.* (27). The ratio of differently phosphorylated peptides was determined using the radioactive tracer.

SDS/PAGE. SDS/PAGE was performed according to Laemmli (28) using 12% polyacrylamide gels in a Hoefer minigel apparatus.

Immunoblot Analysis. Immunoblot analysis was performed according to the methods of Burnette (29). Rhodopsin was detected using monoclonal antibody 1D4 or B6-30. Antibody binding was visualized using a secondary antibody conju-

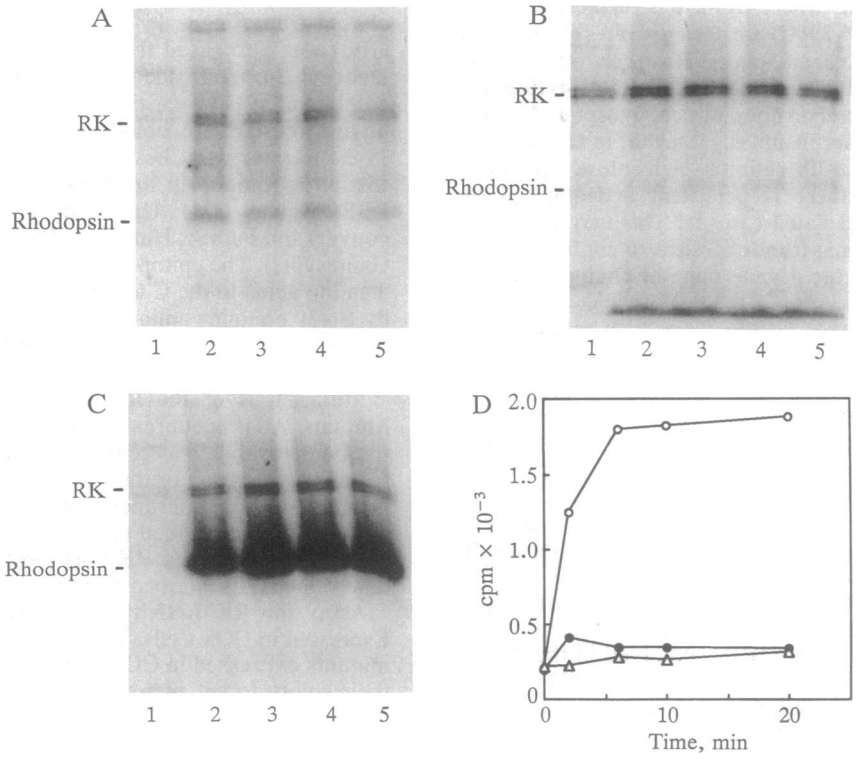

FIG. 1. Time course of phosphorylation of wild-type opsin and rhodopsin expressed in COS cells by RK. COS cell membranes (50 μl) containing wild-type opsin (*A*) or regenerated rhodopsin (*B* and *C*) were added in the dark to a reaction mixture containing 5 μl of ATP (1.6 mM, 1200 cpm/pmol; yielding a final concentration of 75 μM ATP) and 50 μl of purified RK (≈1 μg). This mixture was then incubated at 20°C in the dark (*B*) or under illumination from a 150-W lamp (*A* and *C*). At 0, 2, 6, 10, and 20 min, 20 μl was added to 5 μl of 25 mM EDTA/100 mM dodecyl β-D-maltoside in 50 mM BTP (pH 7.5). This suspension was analyzed by SDS/PAGE. Radiolabeled protein bands were visualized using autoradiography. (*A*) Wild-type opsin. (*B*) Regenerated rhodopsin assayed in the dark. (*C*) Regenerated rhodopsin assayed under illumination conditions. (*A–C*) Lanes: 1, 0 min; 2, 2 min; 3, 6 min; 4, 10 min; 5, 20 min. (*D*) Quantitation of radiolabeled proteins. The amount of radioactivity incorporated into each band was determined as described by Buczyłko *et al.* (25). The radioactivity from the gels visualized in *A–C* and a separate identical experiment was determined. These data represent the average of these two experiments. ●, Dark; ○, light; △, opsin.

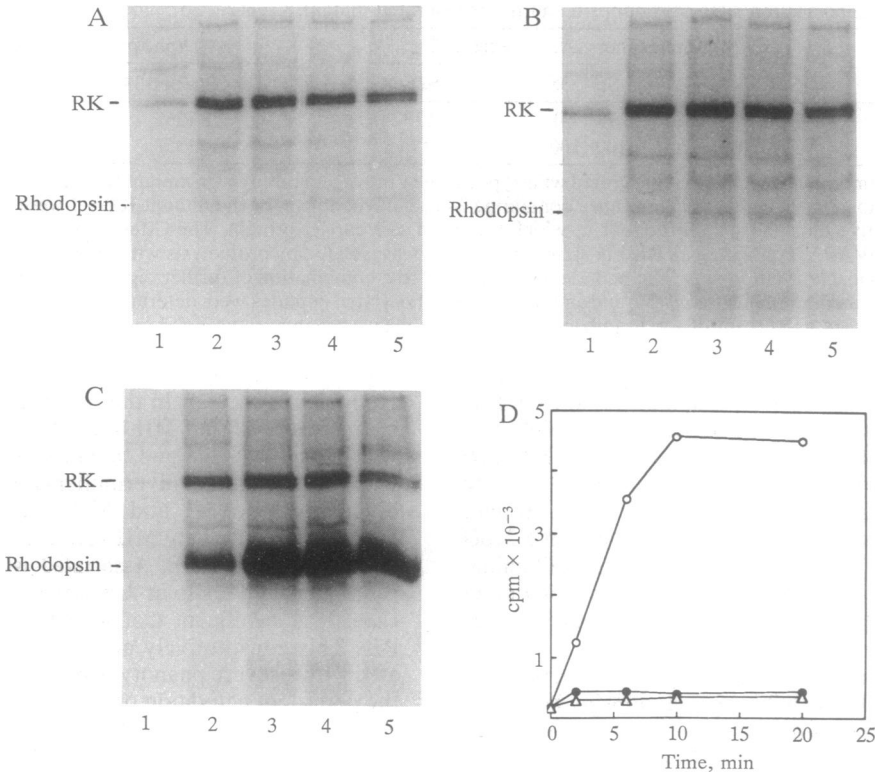

FIG. 2. Time course of phosphorylation of the mutant K296G expressed in COS cells by RK. COS cell membranes (50 μl) containing the mutant opsin without chromophore (*A*) or the opsin regenerated with nPrSB (*B* and *C*) were assayed as described in Fig. 1 and phosphorylation was analyzed by SDS/PAGE and autoradiography. (*A*) K296G opsin without chromophore. (*B*) Regenerated mutant rhodopsin assayed in the dark. (*C*) Regenerated mutant rhodopsin assayed under illumination conditions. (*A–C*) Lanes: 1, 0 min; 2, 2 min; 3, 6 min; 4, 10 min; 5, 20 min. (*D*) Quantitation of radiolabeled proteins as described in Fig. 1. The radioactivity from the gels visualized in *A–C* and a separate identical experiment was determined. The data represent the average of these two experiments. ●, Regenerated in the dark; ○, regenerated in the light; △, mutant opsin.

gated to alkaline phosphatase. The color development system was obtained from Promega.

RESULTS AND DISCUSSION

Rhodopsin in COS Cell Membranes Is Phosphorylated by RK. Wild-type bovine rhodopsin expressed in COS cell membranes was rapidly phosphorylated by RK in a light-dependent manner. The reaction was monitored by SDS/PAGE and autoradiography. As seen in Fig. 1, rhodopsin was phosphorylated only when it had been regenerated with 11-*cis*-retinal and illuminated. Neither opsin (Fig. 1*A*) nor regenerated rhodopsin assayed in the dark (Fig. 1*B*) was phosphorylated by RK. The time-dependent phosphorylation observed in these autoradiograms reflects the autophosphorylation of RK. The autophosphorylation of RK acted as an internal control in these experiments and indicates that RK was active. The time course of the rhodopsin phosphorylation reaction reached a plateau in 5 min (Fig. 1*D*). The data presented here using rhodopsin expressed in COS cells in an *in vitro* reconstitution system are similar to those obtained using native bovine rhodopsin and confirm earlier experiments of Bhattacharya *et al.* (30), who also measured the light-dependent phosphorylation of wild-type rhodopsin expressed in COS cells.

The Constitutively Active Opsin Mutants K296E and K296G Are Not Phosphorylated by RK. It was recently demonstrated that replacing the positively charged Lys at position 296 with either a neutral or negatively charged amino acid results in a constitutively active opsin molecule (18, 19). That is, G_t is activated by these apoproteins in the absence of chromophore and in the absence of light. It was of interest to determine whether these mutant opsins were also phosphorylated by RK. If the conformation that these mutant opsins assume is equivalent to R*, then we might expect that they would be phosphorylated by RK. However, as seen in Figs. 2 and 3, these opsins were not phosphorylated by RK. The time-dependent autophosphorylation of RK, however, indicates that RK was active. This result suggests that there are at least two active conformations of rhodopsin, one that binds and activates G_t and a second distinct conformation that binds RK.

The data presented in Fig. 2*C* suggest that the presence of chromophore in the binding pocket of the mutant opsin is important in forming an active conformation that can bind and act as a substrate for RK. The mutant K296G can be regenerated with the chromophore nPrSB to form a visual

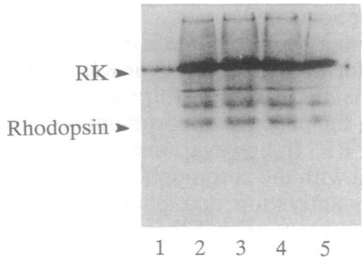

FIG. 3. Time course of phosphorylation of the mutant K296E expressed in COS cells by RK. COS cell membranes (50 μl) containing the mutant opsin without chromophore were assayed as described in Fig. 1, and phosphorylation was analyzed by SDS/PAGE and autoradiography. Lanes: 1, 0 min; 2, 2 min; 3, 6 min; 4, 10 min; 5, 20 min.

Table 1. Phosphorylation sites of rhodopsin and the regenerated mutant K296G

	Monophosphorylated species, %			Diphosphorylated species, %	
Opsin	Ser-334	Ser-338	Ser-343	Thr-336/Ser-338	Ser-338/Ser-343
Rhodopsin	8 (3)	73 (74)	19 (24)	8 (26)	82 (74)
K296G	Trace	100 (100)	Trace	30 (35)	70 (65)

COS cell membranes containing regenerated wild-type opsin or the regenerated opsin mutant K296G were phosphorylated by RK. ^{32}P-phosphorylated COS membranes containing 2–4 μg of rhodopsin or the mutant K296G were mixed with 1 mg of nonradioactive phosphorylated rhodopsin, which was used as a carrier protein. The C-terminal peptide, ^{330}DDEASTTVSKTETSQVAPA, containing all RK phosphorylation sites was isolated by proteolysis with endoproteinase Asp-N. For mapping the phosphorylation sites, the peptide was subjected to a combination of further digestions with thermolysin and trypsin as described by Ohguro *et al.* (27) and amounts of phosphorylated peptides were determined with a radioactive tracer. Data are expressed as percent of total radioactivity from two measurements, the second in parentheses.

pigment that has a wild-type spectrum, is inactive in the dark, and only when illuminated activates G_t (31). As seen in Fig. 2*C*, regenerated K296G was phosphorylated in the light and not in the dark. The kinetics of phosphorylation (Fig. 2*D*) were similar to that of wild-type rhodopsin (Fig. 1*D*). The phosphorylation reaction reached a plateau within 10 min. The binding of chromophore, which forces the mutant K296G into an inactive conformation, and the subsequent illumination of the chromophore allow the mutant opsin to adopt an active conformation(s) that can activate G_t and bind RK, thus resembling wild-type rhodopsin.

Comparison of the kinetics of the phosphorylation reaction of regenerated K296G and wild-type rhodopsin, however, suggests that the reactions may not be identical. To investigate this apparent discrepancy further, we determined whether the sites of phosphorylation were similar in both the regenerated mutant opsin and wild-type rhodopsin. The data presented in Table 1 indicate that the phosphorylation reaction in the regenerated mutant is not identical to that of rhodopsin. When the phosphorylation sites of wild-type rhodopsin expressed in COS cells were determined using a combination of proteolysis by endoproteinase Asp-N, thermolysin, and trypsin, it was found that the distribution of phosphorylation sites in both mono- and diphosphorylated species was identical with the distribution recently determined in native bovine rhodopsin (27). However, in the illuminated regenerated mutant K296G, the initial phosphorylation site was found only at Ser-338. The diphosphorylated species Ser-338/Ser-343 was also decreased in the mutant (70%) as compared to wild-type rhodopsin (82%), with a significant increase in the amount of the Thr-336/Ser-338 diphosphorylated species. The decreased incorporation of phosphate into Ser-343 and Ser-334 may reflect the fact that the complex that the mutant K296G forms with RK is different from that of wild-type rhodopsin. It has been suggested that the fidelity of phosphorylation at multiple sites is a reflection of the low affinity between RK and the C-terminal region (26). However, the position of the catalytic domain of RK in relationship to rhodopsin will also help determine the specific amino acids in the C-terminal region that are phosphorylated.

The Conformation of Rhodopsin that Activates G_t Is Distinct from the Conformation Required for RK Binding and Activity. Mutant opsins that are constitutively active, in that they activate G_t in the absence of chromophore and light, are not substrates for RK. If, however, one of these mutants, K296G, is regenerated with the chromophore nPrSB and illuminated, then both G_t activation and phosphorylation by RK are observed. This suggests that there are two active conformations of rhodopsin. One conformation is mimicked by the mutants K296G and K296E where the salt bridge between Glu-113 and Lys-296 has been disrupted and this opsin can activate G_t. The second active conformation that binds and activates RK requires the presence of the all-trans chromophore in the binding pocket. The notion that there are two active conformations, one capable of activating G_t and one capable of binding and activating RK, is also supported by Hofmann *et al.* (32). In the presence of all-*trans*-retinal and opsin at pH 7.5, an MII-like intermediate was formed that was phosphorylated and bound arrestin but did not interact with G_t. In addition Pulvermüller *et al.* (33) found that RK will bind both the MI and MII spectral intermediates of rhodopsin, while only MII activates G_t.

The Mutant K296E, Associated with RP, May Cause Degeneration by Persistent Activation of the Transduction Cascade. It is significant that K296E is not phosphorylated by RK. This constitutively active rhodopsin mutant is associated with a severe phenotype of RP. It was hypothesized that the persistent activation of the signaling pathway may cause RP by depleting the photoreceptor of RK and arrestin, two proteins used to inactivate R*. In this paper, we demonstrate that K296E is not phosphorylated by RK in our *in vitro* assay. This suggests that *in vivo* this mutant opsin may not deplete the inactivation pathway but rather exerts its effects by persistent activation of G_t. This persistent activation of the G-protein-mediated biochemical cascade may mimic that of long-term exposure to continuous illumination. Fain and Lisman (34) have proposed that continuous real or equivalent light produce outer segment degeneration "by interfering with certain circadian processes."

There Are at Least Two Classes of Constitutively Active Mutants of G-Protein-Linked Receptors. In addition to rhodopsin, mutations in several other G-protein-linked receptors result in constitutive activity (35). These constitutively active receptors can be grouped into at least two classes. The first group, represented by the rhodopsin mutants K296E and K296G, can activate a G protein in the absence of light or in the absence of an agonist but cannot bind and act as a substrate for a kinase. For instance, the rhodopsin mutants K296E and K296G can activate G_t but are not phosphorylated by RK. The second class of constitutively active receptors, represented by a mutant of the α_2-adrenergic receptor, can both activate a G protein and serve as substrate for a kinase. For the α_2-adrenergic receptor, a mutation in the third intracellular loop results in a protein that in the absence of agonist can both activate a receptor-specific G protein as measured by agonist-independent inhibition of adenyl cyclase and can bind and act as a substrate for the β-adrenergic receptor kinase (36). The different types of constitutively active G-protein-linked receptors may be important in understanding the activation of wild-type receptors.

Note Added in Proof. A possible mechanism for constitutive activation has recently been proposed (37).

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