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Farnesylation of Retinal Transducin Underlies Its Translocation during Light Adaptation

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Summary

G proteins are posttranslationally modified by isoprenylation: either farnesylation or geranylgeranylation. The γ subunit of retinal transducin (Tα/Tβγ) is selectively farnesylated, and the farnesylation is required for light signaling mediated by transducin in rod cells. However, whether and how this selective isoprenylation regulates cellular functions remain poorly understood. Here we report that knockin mice expressing geranylgeranylated Tγ showed normal rod responses to dim flashes under dark-adapted conditions but exhibited impaired properties in light adaptation. Of note, geranylgeranylation of Tγ suppressed light-induced transition of Tβγ from membrane to cytosol, and also attenuated its light-dependent translocation from the outer segment to the inner region, an event contributing to retinal light adaptation. These results indicate that, while the farnesylation of transducin is interchangeable with the geranylgeranylation in terms of the light signaling, the selective farnesylation is important for visual sensitivity regulation by providing sufficient but not excessive membrane anchoring of Tβγ.

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Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/47/4/529/DC1/.

Introduction

Protein isoprenylation is an important posttranslational lipid modification. One of two types of isoprenoids, farnesyl (C15) and geranylgeranyl (C20), is linked via a thioether bond to the C-terminal cysteine residue of a variety of cellular proteins, such as heterotrimeric G protein γ subunits and small G proteins (Gautam et al., 1998; Fu and Casey, 1999; Sebti and Der, 2003). The γ subunits of retinal rod transducin (Tγ, also called Gγ1) (Fukada et al., 1990; Lai et al., 1990) and cone transducin (Gγ8) (Ong et al., 1995) are farnesylated, whereas most other subtypes of heterotrimeric G protein γ subunits are geranylgeranylated (Matsuda et al., 1998; Matsuda and Fukada, 2000). Similarly, among members of the small G protein superfamily, Ras proteins are farnesylated (Casey et al., 1989), while Rho and Rab proteins are geranylgeranylated. It has been clearly demonstrated that farnesylation or geranylgeranylation of these G proteins is indispensable for their functions (Casey et al., 1989; Fukada et al., 1989, 1990; Jackson et al., 1990; Cox et al., 1992). Also, the selective isoprenylation has attracted considerable attention from the functional standpoint (Fu and Casey, 1999; Sebti and Der, 2003). Generally, C-terminal sequences with the CAAX motif (C and A being cysteine and aliphatic amino acids, respectively) direct protein isoprenylation, and the X residue is the major determinant for the alternative isoprenylation catalyzed by either farnesyltransferase or geranylgeranyltransferase type I (Fu and Casey, 1999). Following isoprenylation, the last three amino acids are cleaved by the type II CAAX prenyl endopeptidase (RCE1) (Kim et al., 1999; Otto et al., 1999), and the newly exposed carboxyl group of the prenylcysteine residue is methylesterified by the isoprenylcysteine carboxyl methyltransferase (Icmt) (Dai et al., 1998; Bergo et al., 2001) (Figure 1A).

For transducin, farnesylation of recombinant Tβγ in insect cells can be replaced by geranylgeranylation by coexpressing Tβ and mutant Tγ having the C-terminal sequence of CVIL (in place of CVIS for farnesylation), which directs geranylgeranylation (Matsuda et al., 1998; Myung et al., 1999) (S74L mutation, Figures 1A and 1B). Under in vitro conditions, transducin still functions when the farnesyl is replaced by the geranylgeranyl (and vice versa for other heterotrimeric G proteins; see Matsuda et al., 1998; Myung et al., 1999; Fogg et al., 2001), though with some difference in activity. On the other hand, transducin reconstituted with unmodified Tγ (i.e., neither farnesylated nor geranylgeranylated) exhibits essentially no GTP binding activity (Fukada et al., 1989, 1990). Likewise, isoprenylation of a small G protein Ras is essential for its transforming activity (Casey et al., 1989; Jackson et al., 1990; Cox et al., 1992), for which the farnesyl and geranylgeranyl are functionally interchangeable (Cox et al., 1992). However, in spite of the widely accepted role of the modifying lipid as a membrane anchor, Ras requires selective farnesylation for its growth function (Cox et al., 1992). Thus, selective isoprenylation either by the farnesyl or by the geranylgeranyl has raised a long-standing question of how the isoprenoid attached covalently to G proteins regulates intracellular G protein-mediated signaling.

To study the functional role of the selective farnesylation of transducin more closely, we have generated a knockin mouse line in which rod transducin was geranylgeranylated instead. We have found that the farnesyl-to-geranylgeranyl replacement in Tγ markedly affects light adaptation of the mouse retina, without affecting the photoresponse of rods in the dark-adapted state. At the cellular level, this physiological phenotype is associated with a slowdown of the massive light-dependent translocation of Tβγ, which is one of the important cellular processes underlying light adaptation. These results provide evidence for the physiological importance of the selective farnesylation versus geranylgeranylation in G protein signaling.

Results

Generation of S74L Knockin Mice

We produced knockin mice with the S74L mutation in Tγ by targeting the vector harboring the S74L mutation to the *Tγ* gene (Figure 1B). The targeted embryonic stem (ES) cell lines were cloned and used for production of chimeric mice (see Figures S1A and S1B in the Supplemental Data available online). After the heterozygous mice were established, the *neo* gene was deleted from the recombinant allele by Cre-*loxP*-mediated recombination (Figures 1B and 1C).

The retinal morphology of the knockin mice homozygous for the S74L mutation (S74L/S74L) was normal, with unaltered thickness of each retinal layer and no symptom of retinal degeneration even in 6-month-old animals (Figures 5A and 5B). Expression levels of retinal proteins in +/S74L and S74L/S74L mice were indistinguishable from those of wild-type (Figure 1D).

Molecular Characterization of Transducin in S74L Knockin Mice

To examine the molecular structure of Tγ, rod outer segment membranes were isolated from wild-type and S74L/S74L mice and exposed to room light for induced formation of a ternary complex composed of transducin and light-activated rhodopsin. Wild-type transducin was extracted as a soluble form by mixing the irradiated membrane suspension with 10 μM GTPγS, which led to dissociation of Tα-GTPγS and Tβγ from light-activated rhodopsin (Kuhn, 1980). In reverse-phase HPLC analysis of the GTPγS-extract, we detected two major peaks, one containing Tγ and the other Tα (Figure 2A), while Tβ was adsorbed to the column (Fukada et al., 1994). Tγ in the first peak showed a molecular mass of 8316.1 (Figure S2A), in good agreement with the calculated mass of the fully processed (i.e., farnesylated and methylated) form of mouse Tγ (molecular mass of 8315.6). Top-down analysis of isolated Tγ using the electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) (Kassai et al., 2005) demonstrated the covalent modification of Tγ by the farnesyl and methyl groups within the extreme C-terminal region (Pro58-Cys71, Figures S2C and S2E), as predicted from the CAAX signal (CVIS) directing farnesylation/methylation at the C-terminal Cys71. On the other hand, from S74L/S74L mice, only a trace of soluble Tγ was detected in the GTPγS extract (Figure 2C), whereas Tα was detected at a level comparable to that from wild-type mice (Figure 2A). In order to analyze the mutant Tγ that had remained in the membrane (as a Tβγ complex), we extracted mutant Tβγ by mixing the GTPγS-treated membranes with bacterially expressed phosducin. Phosducin stably forms a soluble complex with farnesylated Tβγ (Lee et al., 1987; Kuo et al., 1989b; Tanaka et al., 1996) and also with its geranylgeranylated form (Matsuda and Y.F., unpublished data; see also Figure 6E). In the HPLC analysis of the phosducin-extracted sample from S74L/S74L mice, we found that Tγ was eluted at a position (retention time = 28.4 min) slightly later than wild-type Tγ (25.6 min), and its molecular mass of 8383.3 agreed well with a calculated value of the geranylgeranylated and methylated form of mouse Tγ (8383.7; Figure 2D and Figure S2B). Detailed analysis of mutant Tγ using ESI-MS/MS confirmed the presence of the geranylgeranyl and methyl groups in the C-terminal fragment (Pro58-Cys71, Figures S2D and S2F), most probably attached to Cys71. The N-terminal amino acid sequence of mutant Tγ was verified to be identical to wild-type starting from Pro2 (Fukada et al., 1990). The phosducin extract prepared similarly from wild-type mice contained an undetectable level of Tγ, supporting stoichiometric quality of our analysis.

We next compared the activities of farnesylated and geranylgeranylated Tβγ by measuring the initial velocity of GTPγS binding in the retinal lysates prepared from wild-type and S74L/S74L mice. Upon exposure to a light flash of varying intensity at 4°C, geranylgeranylated transducin in S74L/S74L retinal lysate exhibited 1.3- to 1.9-fold higher initial velocity than farnesylated

transducin in the wild-type lysate (Figure 3A). This observation is consistent with our previous in vitro reconstitution experiments, in which purified wild-type Tα reconstituted with mutated (S74L) Tβγ exhibited a rate constant for GTPγS binding about 3-fold higher than that with wild-type (farnesylated) Tβγ (Matsuda et al., 1998). In the present study, however, the difference in the reaction rate between the two genotypes was blunted at 37°C when the membrane concentration was increased (from 0.5 μM to 3 μM in terms of rhodopsin concentration, Figure 3B). In the dilute homogenate in vitro (e.g., 0.5 μM rhodopsin), farnesylated Tβγ may be dissociated partly from outer segment membranes, hence reducing the coupling efficiency between rhodopsin and transducin and resulting in a lower velocity of GTPγS binding compared with geranylgeranylated Tβγ, which is associated more stably with the membrane (see Discussion).

Retinal Electrophysiology of S74L Knockin Mice

Rod photoreceptors are capable of high sensitivity and light adaptation, allowing them to respond to single photons (Baylor et al., 1979) and to regulate sensitivity in response to background illumination (Pugh and Lamb, 2000; Burns and Baylor, 2001; Fain et al., 2001; Arshavsky et al., 2002). We used the suction pipette method to record the flash responses from individual rods in the dark-adapted state. Light flashes of increasing intensities elicited similar sets of photoresponses from wild-type and S74L/S74L rods (Figure 4A). No significant difference was observed between the two lines both in the maximal response amplitude (r_{max}) and in the light intensity that evoked a half-saturating response (i_o) (Table 1). The dim-flash responses were also indistinguishable in kinetics (Figure 4B, and t_p and t_i in Table 1) and in amplitude as evoked by a single photon (a in Table 1, calculated as the response ensemble variance-to-mean ratio, Sung et al., 1994). These results indicate that rod response properties are not affected by the type of isoprenyl modification of Tγ under dark-adapted conditions, consistent with the data on GTPγS binding at high membrane concentration (Figure 3B).

We next examined a change in the rod photosensitivity of S74L/S74L mice after prolonged light adaptation by measuring the full-field electroretinogram (ERG) a-wave, well established to reflect the light response of photoreceptors (predominantly rods in mouse). To characterize the amplification of rod phototransduction, the leading edge of the a-wave was fitted with a theoretical trace calculated according to the Lamb-Pugh model (Lamb and Pugh, 1992; Arshavsky et al., 2002) (see Experimental Procedures). An important parameter in the fit is the amplification constant (A), which represents the gain factor in the rising phase of rod phototransduction and is therefore a reflection of light sensitivity. We collected a set of ERG responses from a fully dark-adapted animal and calculated A_{dark}. Afterward, the animal was exposed to adapting light (100 lux or 250 lux) for 10 min and then subjected to a second round of ERG recording and $A_{adapted}$ was derived (Figure S3). No statistically significant difference in the fully dark-adapted A (A_{dark}) value (as well as the parameters a_{max} and t_{eff}; see Experimental Procedures) was observed between wild-type and S74L/S74L animals (Table S1, dark). This finding is consistent with the above results from single-cell recordings (Figures 4A and 4B) and indicates that the mutation has no measurable effect on the activation phase of phototransduction in the dark-adapted rod. When kept in the dark during the sham "adaptation" period (control), wild-type and S74L/S74L mice exhibited indistinguishable values of $A_{control}/A_{dark}$ (Figure 4C), which were slightly lower than 1.0, probably owing to adaptation to the test flashes in the ERG recordings, as described previously (Sokolov et al., 2002). After 10 min exposure to the 100 lux adapting light, $A_{adapted}$ in the wild-type mice was reduced to less than 20% of A_{dark}, indicative of light adaptation; in S74L/S74L mice, on the other hand, the reduction in $A_{adapted}$ due to the 100 lux adapting light was much smaller (Figure 4D). This difference due to the S74L mutation was largely independent of the test flash intensity in the range of 0.005–0.05 relative to the saturating flash intensity (Figure 4D). When the intensity of the 10 min adapting light was raised to 250 lux, however, $A_{adapted}/A_{dark}$ even in

S74L/S74L mice decreased significantly to a level almost identical to that of wild-type mice (Figure 4D). These results indicate that the S74L mutation markedly impairs light adaptation under the 100 lux light but not so much under a brighter light (250 lux).

Impaired Translocation of Transducin in S74L/S74L Mice

To explore the possible mechanism underlying the impaired light adaptation in S74L/S74L mice, we investigated immunohistochemically the light-dependent translocation of transducin (Hardie, 2002; Sokolov et al., 2002; and references cited therein) under lighting conditions identical to those used for the ERG recordings. In the dark-adapted state, both Tα and Tγ immunoreactivities were predominantly detected in the outer segment layer of wild-type and S74L/S74L retinas (Sokolov et al., 2002) (Figures 5C, 5F, 5I, and 5L). After a 10 min exposure to the 100 lux adapting light, Tβγ in wild-type mice translocated to the inner regions of the photoreceptor layer, including the inner segment, the perinuclear region, and the synaptic terminal (Figure 5J). In contrast, in the S74L/S74L mouse retina, only a trace of Tγ immunoreactivity was detected in these inner regions after the 100 lux light adaptation (Figure 5M). In both mouse genotypes, minimal movement of Tα was observed under the same adaptation conditions (Figures 5D and 5G). With a 10 min exposure to a brighter (250 lux) light, the movement of Tβγ in wild-type retina was slightly more enhanced than under the 100 lux light (Figures 5K and 5P); in S74L/S74L retina, however, a large part of the Tβγ signals still remained in the outer segment layer (Figures 5N and 5P), a profile that is very similar to the distribution of wild-type Tβγ in the dark-adapted state (Figures 5I and 5P and Figure S4B). At 250 lux, Tα now showed considerable translocation to the inner region of the photoreceptor layer, and the localization profiles of Tα immunoreactivity were very similar in the two genotypes (Figures 5E and 5H). Quantitative analysis of these data (Figures 5O and 5P and Figure S4) supports that the translocation of Tβγ is markedly slowed down by the S74L mutation and that the light-dependent translocation of Tα is not affected by the mutation.

We then asked whether geranylgeranylated Tβγ may be solubilized in vivo by the regulatory protein phosducin, which associates tightly with Tβγ and reduces its affinity for outer segment membranes (Lee et al., 1987; Kuo et al., 1989b; Tanaka et al., 1996; Sokolov et al., 2004). By taking advantage of “native” PAGE capable of detecting protein complexes (Williams and Reisfeld, 1964; Chen and Lee, 1997), we pursued the light-dependent change in levels of Tβγ and Tβγ-phosducin complex in the soluble fraction of the retinal homogenate. A 10 min exposure of wild-type mice to 250 lux light remarkably increased the amounts of both Tβγ and Tβγ-phosducin complex in the retinal soluble fraction (Figures 6A and 6B). In contrast, they were almost undetectable in the soluble fraction prepared from S74L/S74L mice, irrespective of light (Figures 6A and 6B), indicating that geranylgeranylated Tβγ remained in the membrane even when light-activated Tα became soluble in the mutant retina (Figure 6C). An in vitro phosducin titration experiment demonstrated that a higher dose of exogenously added phosducin was required for extracting geranylgeranylated Tβγ from the light-exposed outer segment membranes of S74L/S74L mice (Figure 6E, solid circles). This property provides a striking contrast to that of farnesylated Tβγ exhibiting high solubility even in the absence of added phosducin (Figure 6E, open circles). It should be noted that phosducin predominantly localizes in the inner regions of the photoreceptor cells (Kuo et al., 1989a; Sokolov et al., 2004), and the molar ratio of phosducin to transducin in the outer segment is supposed to be lower than one-tenth (Sokolov et al., 2004). Application of this ratio to our titration experiment gave an estimated value of ~1.2 pmol phosducin, an amount that should be insufficient for solubilizing geranylgeranylated Tβγ (Figure 6E). Hence the suppression of light-dependent translocation of Tβγ in S74L/S74L rods is attributable to the higher affinity of geranylgeranylated Tβγ for the membrane. It appears that the selective farnesylation of Tγ provides Tβγ with the proper (sufficient but not excessive) membrane affinity, allowing Tβγ

to stay with Tα in the outer segment in the dark and to translocate to the inner region in the light.

Discussion

In this study, we have investigated the physiological significance of the selective farnesylation of transducin by using the S74L mutant mice, in which the farnesyl of Tγ was replaced by the geranylgeranyl without any change in amino acid sequence in its mature form. Therefore, the molecular and physiological phenotypes observed for the mutant mice can be attributed solely to the change in the C-terminal isoprenyl modification. In the in vitro experiment with the retinal lysate, farnesyl-to-geranylgeranyl replacement had no effect on the initial velocity of GTPγS binding at 37°C at the higher rhodopsin concentration (3 μM, Figure 3B). Likewise, we observed no measurable effect of the specific isoprenylation on the parameters of the rod photoresponse under dark-adapted conditions (Figures 4A and 4B). It is interesting to note that the rate of GTPγS binding of wild-type transducin was significantly lower than that of S74L mutant transducin in the dilute homogenate (rhodopsin concentration of 0.5 μM) at 4°C (Figure 3A). Such a difference between in vitro and in vivo experiments may be accounted for by the extremely dense packing of the signal-transducing proteins in between rod disk membranes in vivo (Burns and Baylor, 2001; Calvert et al., 2001). The pseudo-two-dimensional array of the light-signaling proteins intrinsically restricts their motion along the Z axis, and this limitation of the protein movement could compensate for the slower GTPγS binding of farnesylated (versus geranylgeranylated) transducin, which was observed in the dilute membrane suspension (Figure 3A).

The light-dependent translocations of various photoreceptor proteins have been reported to contribute to light adaptation on a long timescale not only in vertebrates (Sokolov et al., 2002) but also in *Drosophila* (Bahner et al., 2002), although the precise mechanisms for the translocations remain to be elucidated (Mendez et al., 2003; Zhang et al., 2003; Giessl et al., 2004; Lee and Montell, 2004). In the case of crayfish photoreceptor, the Gq-type G protein undergoes light-induced translocation between rhabdomeric membranes and cytoplasm, and this solubilization of the G protein determines its subcellular localization (Terakita et al., 1996, 1998). Our present results indicate that wild-type Tβγ (but not geranylgeranylated Tβγ) was solubilized from the cellular membranes in response to the adapting light (Figure 6) and that selective farnesylation of Tγ facilitates Tβγ translocation within the rod cells (Figure 5 and Figure S4). The translocation of Tβγ should contribute to the decrease in rod sensitivity, because a declined level of Tβγ in the outer segment should reduce the efficiency of Tα already activated during the light adaptation to be reprimed for activation through reassociation with Tβγ. The slowdown of the light-dependent translocation of Tβγ by the S74L mutation, accompanied by a lower degree of light adaptation exhibited by the mutant rods, strongly supports the notion that selective isoprenylation of Tγ plays a critical role in this adapting function. Of note, the difference between wild-type and mutant mice becomes much smaller under the more intense adapting light of 250 lux (Figure 4D); however, this can be attributed to the translocation of Tα, which occurs similarly in both genotypes under the stronger light (Figure 5O). In our present study, the reduction in the amplification constant of the ERG a-wave (Figure 4D) was not predicted fully from the amount of translocated transducin (Figure 5). The difference may be ascribed to an adaptation mechanism(s) other than, but in parallel with, transducin translocation, such as the regulation by calcium ions and visual pigment phosphorylation (reviewed in Pugh et al., 1999).

We observed only a marginal effect of the S74L mutation in Tγ on the light-dependent translocation of Tα (Figure 5O), corroborating the data showing that, in both genotypes, Tα becomes highly soluble after light activation (Figure 6C) with or without added phosducin (Figure 6D). These observations together suggest that diffusion of solubilized transducin

subunits may underlie their light-dependent translocation to the inner parts of rods, where phosducin associates with Tβγ for stabilization (Obin et al., 2002). This model is consistent with the aberrant localization of transducin in another type of mutant mice that express unmodified Tγ in the rod cell (H.K., A.A., K.N., K.N., M.K., H.I., Y.S., T.O., and Y.F., unpublished data): Tβγ in these mutant mice with unmodified Tγ displays extremely high solubility and is located diffusely in the inner region of the rod cells even in the dark-adapted state, demonstrating an essential role of Tγ farnesylation for proper localization and redistribution of transducin. The unique solubility of transducin among G proteins is now to be highlighted because the retina-specific N-terminal lipid modification of Tα (less hydrophobic than pure *N*-myristoylation of other Gαs) (Kokame et al., 1992) may likewise contribute to the regulation of light-dependent Tα movement. Interestingly, the γ subunit of cone transducin (Gtα2/Gβ3γ8) is also selectively farnesylated at the C terminus (Ong et al., 1995). In response to a discrete range of light intensities, cone transducin may undergo light-induced translocation to regulate cone sensitivity as well, though the degree of its contribution may be different from that in rods or may vary among species (Kennedy et al., 2004).

Among small G proteins, the transforming activity of Ras is promoted not only by the farnesyl but also by the geranylgeranyl modification, demonstrating the interchangeability of these two isoprenoids with respect to this particular function (Cox et al., 1992). However, in contrast to farnesylated Ras, geranylgeranylated Ras exhibits potent growth inhibition of cultured NIH3T3 cells (Cox et al., 1992), indicating that normal Ras function does specifically require its modification by the farnesyl. It is tempting to speculate that a difference in common molecular events such as protein translocation imparted by the two isoprenyl groups may underlie functional differences between farnesylated Ras and geranylgeranylated small G proteins.

Finally, the S74L/S74L mutant mouse is unique in that the modifying lipid is replaced without involving any alteration in the primary structure of the protein. In this way, we were able to demonstrate unequivocally a new role of lipid-modification in proteins, in this case the physiological importance of selective farnesylation over geranylgeranylation in regulation of the G protein-mediated signal transduction.

Experimental Procedures

Generation of Knockin Mice

The animal experiments were conducted in accordance with the guidelines of the University of Tokyo. Mouse genomic fragments containing the protein-coding region of the *Tγ* gene were isolated by screening a λ FIX II genomic DNA library prepared from the 129/Sv mouse inbred strain (Stratagene). The isolated clones were used for constructing the targeting vector carrying the S74L mutation together with a Hinf I restriction site used for genotyping (Figure 1B). The S74L mutation was introduced to the targeting vector by PCR-based site-directed mutagenesis and was verified by DNA sequencing. A *loxP*-flanked *neo* gene under the control of the HSV-*tk* promoter was inserted into the Kpn I site in the middle of the intron between exons 3 and 4 as a positive selection marker. At the 5′ end of the targeting vector, the diphtheria toxin A fragment (*DT-A*) gene driven by the HSV-*tk* promoter was added as a negative selection marker (Figure 1B and Figure S1A). Cultured E14 ES cells (5.0×10^7 cells) were transfected with 50 μg of the linearized targeting vector by electroporation (BTX Electro Cell Manipulator), followed by selection with G418 treatment (250 μg/ml) 48 hr after the transfection. Altogether, 240 drug-resistant colonies were picked up on day 8 of the selection, and the genomic DNA was subjected to Southern blot analysis for confirmation of homologous recombination (Figure S1B). Introduction of the S74L mutation was also verified by Hinf I digestion of the PCR fragments amplified with a set of primers (5′-TAGCT CAAGG GTCAG CTTTC-3′ and 5′-CTGCT TCTAA AGCAG GGTTC-3′, Figures S1E and S1F) and by DNA sequencing. The targeted ES clones isolated were microinjected into C57BL/6J blastocysts to generate chimeric

mice, and the chimeric males thus generated were mated with C57BL/6J females. Germ-line transmission was ascertained by both Southern blot and PCR analysis of the genomic DNA prepared from these litters in the same way as described for the screening of the ES cells. After heterozygous animals were obtained, the *neo* gene was deleted by injecting a Cre-expression plasmid into the fertilized eggs obtained by crossing a heterozygous male with C57BL/6J females by in vitro fertilization (Figure 1B and Figure S1C). To examine for excision of the *neo* gene, genomic DNA isolated from the progeny was digested with BamH I/Bgl II restriction enzymes and subjected to Southern blot analysis with the probe indicated in Figure 1B (see also Figure S1D).

Immunoblot

Retinas from wild-type or mutant mice were homogenized in SDS sample buffer containing 10 mM Tris-HCl (pH 6.8 at 25°C), 2% (w/v) SDS, 50 mM dithiothreitol (DTT), 2 mM EDTA, and 6% (v/v) glycerol. Protein concentrations were estimated by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. Proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE) were transferred to PVDF membrane (NT-31, Nihon Eido) and probed with antibodies against Tγ (20 ng/ml, Santa Cruz Biotechnology), Gβ (20 ng/ml, Santa Cruz Biotechnology), Tα (20 ng/ml, Santa Cruz Biotechnology), rhodopsin (1:30,000, raised in our laboratory), and recoverin (1:2000, raised in our laboratory). Bound antibodies were detected by secondary antibodies conjugated with horseradish peroxidase (150 ng/ml, KPL) and visualized by an enhanced chemiluminescence detection system (Western Lightening, NEN).

Purification and Analysis of Mouse Tγ

Rod outer segment membranes were prepared from dark-adapted mouse retinas under dim red light, as follows. Eyes enucleated from dark-adapted mice (older than 8 weeks) were cut into two parts and collected in microtubes (10 eyeballs per tube, 50 eyeballs in total). They were suspended in 800 μl of buffer R (10 mM MOPS-NaOH [pH 7.5 at 4°C], 2 mM $MgCl_2$, 30 mM NaCl, 60 mM KCl, 1 mM DTT, 4 μg/ml aprotinin, 4 μg/ml leupeptin) and centrifuged at 15,000 × g for 5 min. The pellet was suspended again in 800 μl of buffer R containing 40% (w/v) sucrose (final concentration) and shaken vigorously by vortexing for 1.5 min for detachment of the outer segments from cell bodies. The suspension was centrifuged at 15,000 × g for 5 min, and the floating membranes were saved. This floatation procedure was repeated two more times, and the rod outer segment membranes thus isolated were purified by a sucrose stepwise gradient centrifugation as described by Fukada et al. (1994). The membrane suspension was exposed to a room light for 10 min and washed three times with 500 μl of hypotonic buffer A (10 mM Tris-HCl [pH 7.2 at 4°C], 0.5 mM $MgCl_2$, 1 mM DTT, 4 μg/ml aprotinin, 4 μg/ml leupeptin). The washed membranes were incubated in 500 μl of buffer A supplemented with GTPγS (final concentration of 10 μM) on ice for 10 min, and the suspension was centrifuged at 100,000 × g for 20 min to save the supernatant. This extraction procedure with GTPγS was repeated two more times, and the extracts were combined ("GTPγS-extract"). The resultant membrane suspension was mixed with recombinant rat phosducin (Lee et al., 1987; Kuo et al., 1989b) (final concentration of 0.6 μM in 500 μl of buffer A). After incubation on ice for 10 min, the suspension was centrifuged to save the supernatant as described above for the GTPγS extraction. This extraction procedure with phosducin was repeated two more times, and the extracts were combined ("phosducin-extract"). These extracts were injected to a reverse-phase column (Cosmosil $5C_{18}$-P300, 4.6 × 150 mm, Nakalai Tesque) equipped with an HPLC system (model 600E, Waters). The isolated fractions were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager RP, Perseptive Biosystems) to determine the molecular mass by using sinapic acid as a matrix and horse cytochrome c as an internal standard of the molecular mass.

Electrophysiology

Photoresponses were recorded from individual rods with a suction pipette as described by Sung et al. (1994). Briefly, a mouse was dark-adapted overnight and sacrificed by cervical dislocation under dim red light. Subsequent procedures were carried out under infrared illumination. The retina was isolated from an enucleated eye and chopped finely in Leibovitz's L-15 (GIBCO) medium supplemented with DNase I (8 μg/ml, Sigma). Small pieces of the chopped retina were transferred to a recording chamber and perfused continuously at 36°C–38°C with medium bubbled with 95% O_2 and 5% CO_2. A single rod outer segment was drawn into a glass suction electrode and stimulated by flashes (8 ms) of 500 nm, unpolarized light with 10 nm bandwidth. The membrane current was recorded with a current-to-voltage converter, low-pass filtered at 30 Hz (eight-pole Bessel), and digitized with pCLAMP6. The solution filling the suction electrode contained 134.5 mM Na^+, 3.6 mM K^+, 2.4 mM Mg^{2+}, 1.2 mM Ca^{2+}, 136.3 mM Cl^-, 3 mM succinate, 3 mM L-glutamate, 10 mM glucose, 10 mM HEPES (pH 7.4), and 0.02 mM EDTA plus basal medium Eagle (BME) amino acid supplement and BME vitamin supplement (GIBCO). The composition of the perfusion medium was the same as that of the electrode-filling solution, except that 20 mM Cl^- was replaced by equimolar HCO_3^-.

ERGs were recorded from dark-adapted mice anesthetized with 1000 urethane, 20 ketamine, and 2.5 xylazine mixture (in μg/g body weight). The eyes were treated with 0.5% (w/v) tropicamide and 0.5% (w/v) phenylephrine-HCl solution (Mydrin-P, Santen) for dilation of pupils and with 0.4% (w/v) oxybuprocaine-HCl solution (Benoxil, Santen) for surface anesthesia. The anesthetized mouse was placed on a heating plate (NS-TC10, Neuro Science Inc.) kept at 37.5°C in a Faraday cage. ERGs were recorded via two electrodes, one placed on the surface of the cornea as a recording electrode and the other inserted subcutaneously in the forehead as a reference electrode. Two light sources were connected via optic fibers to a ganzfeld for delivering white light to the eyes. One was a xenon lamp (LX-500, Sanso) for steady adapting light, and the other was a xenon short arc flash (SA-200, Nissin Electronic Co.) for test flashes, which at the highest intensity (relative intensity of 1.0, ~ 1.6×10^6 photoisomerizations/rod) saturated the a-wave amplitude. Four subsaturating flashes (relative intensities of 0.005–0.05) were delivered before and 20 min after 10 min exposure to the adapting light of 100 lux. The ERG responses were amplified, band-pass filtered at 0.5–1000 Hz (AVB-21, Nihon Koden), digitized by an analog-to-digital converter (Digidata 1321A, Axon Instruments), and the final forms were stored on a personal computer using Axo-scope software (Axon Instruments). Theoretical analysis of the ERG a-waves was performed as described by Lyubarsky and Pugh (1996) and Sokolov et al. (2002) using the following equation developed from a model for the activation of phototransduction (Lamb and Pugh, 1992; Lyubarsky and Pugh, 1996; Arshavsky et al., 2002):

$$a(t)/a_{\max} = 1 - \exp\left[-\frac{1}{2}\Phi A(t - t_{\text{eff}})^2\right]$$

where $a(t)$ is the response amplitude at time t after the flash, a_{max} is the maximal amplitude of the a-wave at the highest intensity of the test flash, Φ is the number of photoisomerizations per rod produced by the test flash at time $t = 0$, A is the amplification constant proportional to the gain of phototransduction, and t_{eff} is an intrinsic delay in the response onset. The three parameters, a_{max}, A, and t_{eff} characterize the combined contributions of the several steps of the phototransduction cascade in rods (Lamb and Pugh, 1992; Lyubarsky and Pugh, 1996; Arshavsky et al., 2002). The leading edge of each a-wave trace was fitted with the equation above, with A and t_{eff} as free parameters. The amplification constants, A_{dark} and $A_{adapted}$, were calculated from the fitted traces before and after light adaptation, respectively. For several minutes after the light adaptation period, the amplitudes of the a-waves were too small to

determine the $A_{adapted}$ values precisely. Therefore, quantitative analyses after the light adaptation were performed by measuring a set of ERGs (in the dark) 20 min after the offset of the light adaptation (Figure 4, Table S1, and Figure 3). In separate experiments, we estimated temporal changes in $A_{adapted}$ values by measuring ERG responses to constant-intensity flashes that were given with 2–3 min intervals from 5 to 20 min (a waiting period) after the offset of the 10 min adaptation with 100 lux light. Then it was verified that the $A_{adapted}$ values from wild-type and S74L/S74L mice were markedly different from each other at 5 min after the offset of the light adaptation and that the recovery rates of their $A_{adapted}$ values were indistinguishable during the waiting period between the two genotypes keeping the large difference of the $A_{adapted}$ value almost unchanged. These analyses are based on the observations that the a_{max} values were kept constant from 5 to 20 min after small bleaching (H.I., N. Tammitsu, Y. Ueda, and Y.S., unpublished data; Maeda et al., 2005), and hence the $A_{adapted}$ were calculated using the a_{max} value that was determined in each animal by the ERG response to the saturating light given at the end of the waiting period.

Histology

Mouse eyes were dissected and fixed in 4% (w/v) formaldehyde in PBS for 1.5 hr at room temperature under dim red light. At the time of enucleation, a small incision was made in the cornea to allow rapid fixation of the retina. The fixed eyes were cryoprotected over-night with 30% (w/v) sucrose in PBS and embedded in OCT compound (TissueTek). Retinal sections of 10 μm thickness were prepared by using a cryostat (CM3000, Leica) and were collected on gelatin-coated glass slides. Air-dried sections were washed in PBS three times and incubated in PBS containing 0.1% (w/v) Triton X-100 for 15 min. After being washed in PBS three times, the sections were incubated in a blocking solution containing 3% goat normal serum in PBS for 1.5 hr and immunostained by the antibody against Tγ (67 ng/ml, Santa Cruz Biotechnology) or Tα (67 ng/ml, Santa Cruz Biotechnology) overnight at 4°C. Bound antibodies were visualized using Alexa-488 goat anti-rabbit IgG conjugates (2 μg/ml, Molecular Probes), and TO-PRO-3 (0.5 μM, Molecular Probes) was used for staining of cell nuclei. The sections were viewed by a confocal laser-scanning microscope (TCS-NT, Leica).

Analysis of Tβγ-Phosducin Complex Formation

Mice were dark adapted overnight and anesthetized in the same way as described in "Electroretinography." After being exposed to 250 lux light or kept in the darkness, the retinas from both eyes were dissected and homogenized in 160 μl of buffer R. The retinal homogenates were centrifuged at 15,000 × g for 10 min, and the supernatants were mixed with glycerol (final 10%) and bromophenol blue (final 0.01%). Native PAGE of the protein sample was performed as described (Williams and Reisfeld, 1964; Chen and Lee, 1997) in a gradient gel of 10%–20% acrylamide, and the proteins in the gel were immunoblotted using antibodies against Gβ, phosducin (1:10,000) (Kuo et al., 1989a), and Tα (see methods of "Immunoblot").

Effect of Phosducin on Solubility of Tβγ

Rod outer segment membranes were prepared from ten eyeballs, exposed to a room light for 10 min, and washed with hypotonic buffer A as described above. Aliquots of the washed membranes (1/24 volume, in buffer A) were incubated on ice for 10 min in the presence or absence of GTPγS (final concentration of 10 μM) and phosducin (0, 2, 10, 50 pmol). Then the suspension was centrifuged at 100,000 × g for 10 min to save the supernatant, which was subjected to Western blot analysis as described in "Immunoblot." The immunopositive signal intensities of Tα and Tβ were quantified by Image Gauge software (Fujifilm), and those in the supernatant were expressed as percentages of the total amount of Tα and Tβ, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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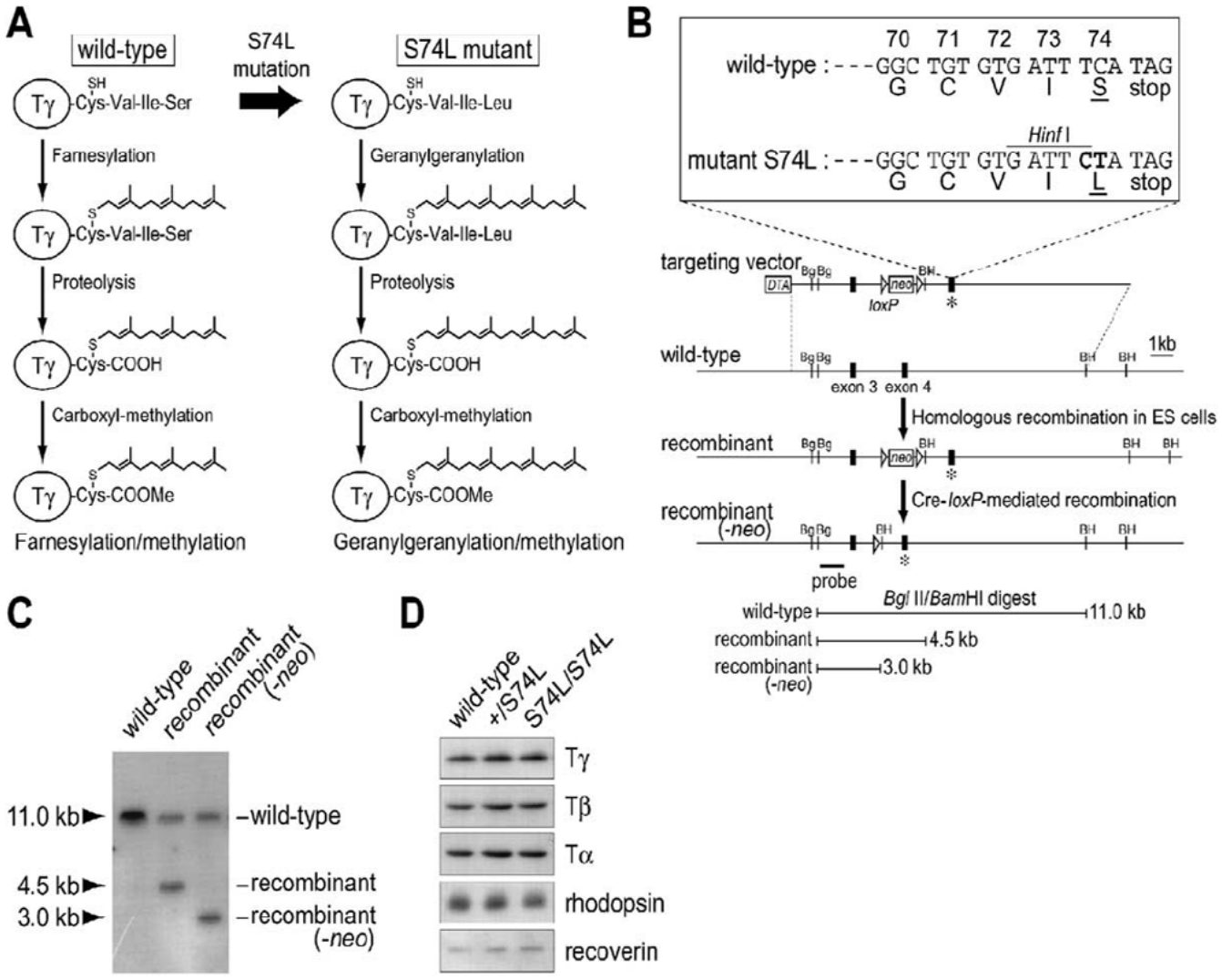

Figure 1. Production of S74L Knockin Mice

(A) Schematic drawing of the C-terminal processing of transducin γ subunit in wild-type and S74L knockin mice.

(B) Targeting strategy for introducing the S74L point mutation into the *T*γ gene. Shown is a region of the *T*γ gene locus that includes exons 3 and 4 (filled boxes), with an asterisk designating the mutated exon 4 that carries the S74L mutation. A thick line indicates the position of a probe used for Southern blot analysis (shown in [C]). Restriction enzyme sites: Bg, Bgl II; BH, BamH I.

(C) Genotyping of S74L knockin mice by Southern blot analysis. DNA isolated from tail biopsies was digested with Bgl II and BamH I restriction enzymes and hybridized with the probe shown in (B). Homologous recombination and excision of the *neo* gene were confirmed by the presence of 4.5 kb and 3.0 kb DNA fragments, respectively.

(D) Comparison of protein levels in retinal homogenates prepared from wild-type, +/S74L, and S74L/S74L mice. In each lane, 20 μg proteins were electrophoresed for blotting of Tγ, Tβ, and Tα; 0.5 μg for rhodopsin; and 10 μg for recoverin.

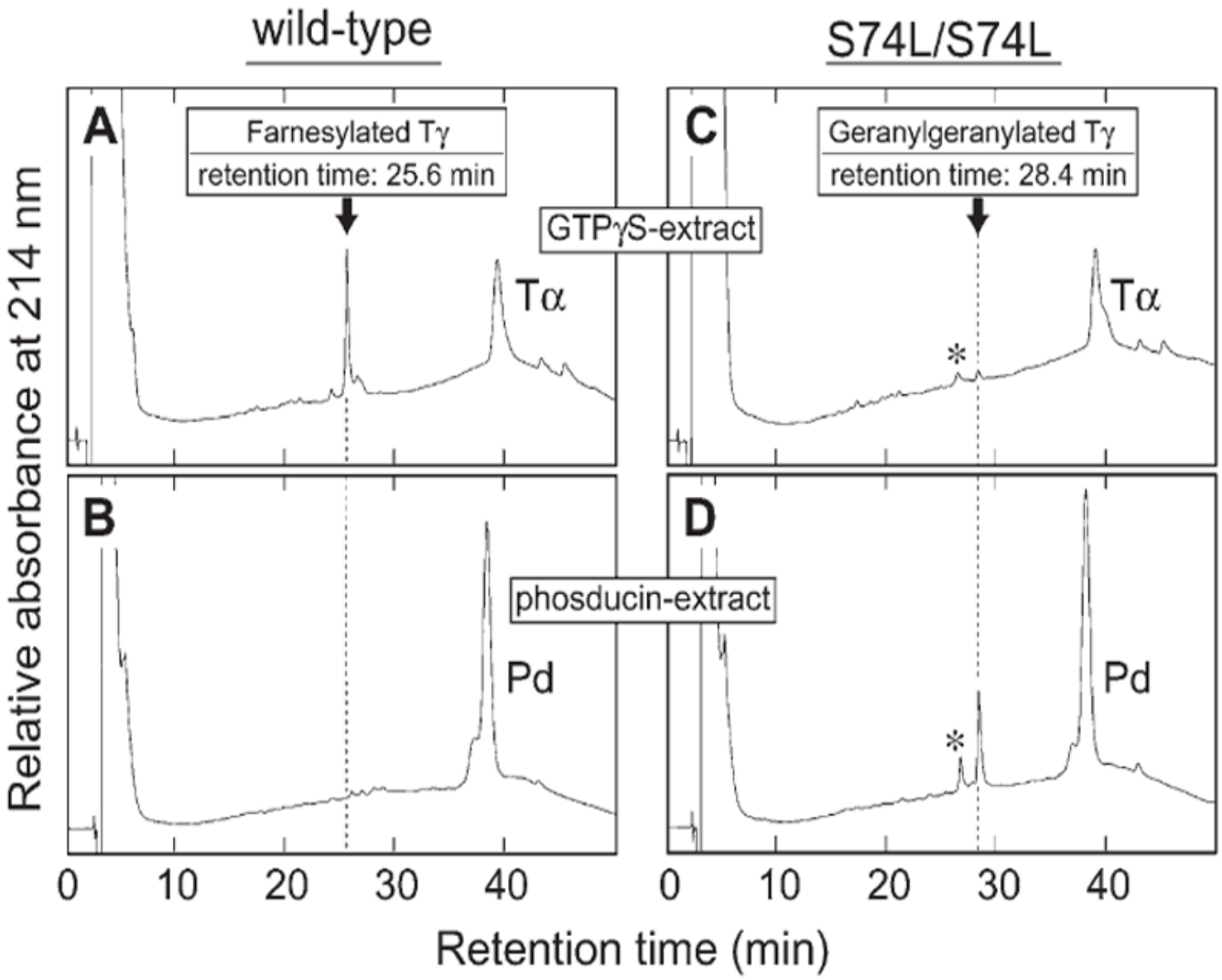

Figure 2. Isolation and Analysis of Tγ

The membrane fraction of retinal outer segments was prepared from wild-type (A and B) and S74L/S74L mice (C and D), and proteins were extracted with 10 μM GTPγS (GTPγS-extract; [A and C]) and subsequently with 0.6 μM phosducin (phosducin-extract; [B and D]). Each extract was injected to a reverse-phase Cosmosil $5C_{18}$-P300 column (Nakalai Tesque) equipped with the HPLC system. Bound materials were eluted with a linear gradient of acetonitrile (30%–80%, 1%/min) in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and the absorbance of the eluate at 214 nm was continuously monitored. The peak fraction containing Tγ immunoreactivity was subjected to MALDI-TOF and ESI-tandem mass spectrometry. The peak marked by an asterisk contained a 20 kDa protein unrelated to Tγ. In this analysis, the amount of geranylgeranylated Tγ recovered from S74L/S74L mice was consistently lower (~70%) than that of farnesylated Tγ from wild-type mice. This was at least partly due to the lower yield of recovery of geranylgeranylated Tγ from the column, probably owing to the increased hydrophobicity.

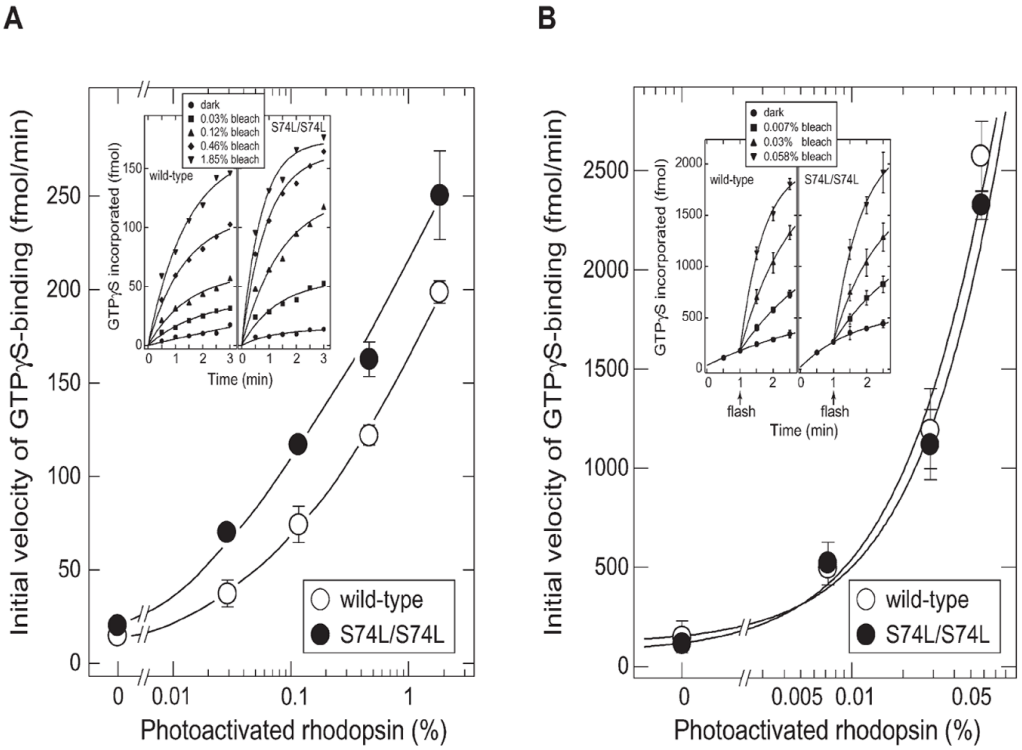

Figure 3. Initial Velocities of Light-Stimulated GTPγS Binding Reaction of Transducin in the Retinal Homogenate

The retinas isolated from wild-type (open circles) and S74L/S74L (closed circles) mice were homogenized in buffer R, and the rhodopsin concentration was determined from the difference spectra before and after complete bleaching of the aliquot solubilized in 1% CHAPS in the presence of 100 mM NH_2OH (Matsuda and Fukada, 2000). Initial velocities of the GTPγS binding to Tα catalyzed by metarhodopsin II were measured either at 4°C or at 37°C in reaction mixtures (50 μl) composed of retinal homogenate in buffer R (final concentration of rhodopsin was fixed to either 0.5 μM [A] or 3 μM [B]), 2 mg/ml ovalbumin, 0.1% CHAPS, and 1 μM [^{35}S]GTPγS (370 GBq/mmol). (A) Initial velocity of GTPγS binding reaction at the rhodopsin concentration of 0.5 μM at 4°C. The reactions were started by the addition of [^{35}S]GTPγS after 2 min interval from exposure of the reaction mixture (minus [^{35}S]GTPγS) to a flash light (>540 nm) with various intensities. (B) Initial velocity of GTPγS binding reaction at the rhodopsin concentration of 3 μM at 37°C. The reaction was started by the addition of [^{35}S]GTPγS in the dark at 37°C, and the assay mixture received a flash of light (>540 nm) after 1 min incubation (arrow in inset). For both experiments, the flash intensity was controlled by calibrated neutral density filters, and the unattenuated flash constantly bleached 7.4% of rhodopsin in the homogenate. The reactions were terminated at various time points by mixing an aliquot (10 μl) of the reaction mixture with 180 μl of buffer W (100 mM Tris-HCl [pH 7.5 at 4°C], 1 mM $MgCl_2$) containing 2.0 mM GTP. [^{35}S]GTPγS bound to Tα was isolated from free [^{35}S] GTPγS by filtrating the samples (190 μl) over 0.45 μm cellulose membranes (type HATF; Millipore) fitted with MultiScreen Assay System (Millipore). Immediately after the filtration, the membranes were washed four times with 200 μl of buffer W, dried, and then counted. Error bars indicate SD (n = 3). Insets, time courses of the GTPγS binding reactions.

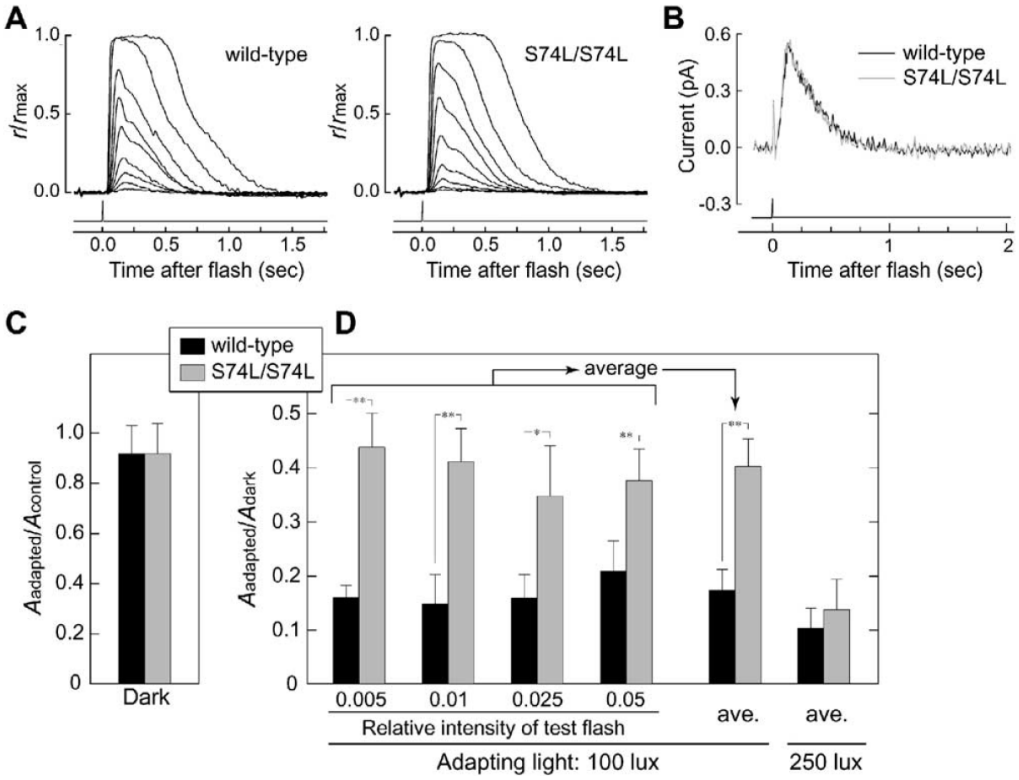

Figure 4. Electrophysiological Analysis of S74L Knockin Mice

(A) Flash responses recorded by a suction electrode from a single dark-adapted rod of wild-type (left) or S74L/S74L (right) mouse to 500 nm flashes of increasing intensities (1.69, 3.45, 6.43, 13.13, 25.02, 51.08, 95.12, 194.21, and 770.35 photons μm^{-2}, respectively). Each trace is the averaged response from multiple flash trials. Stimulus monitor traces are shown below the records.

(B) Dim-flash responses from a dark-adapted rod of wild-type (black trace) or S74L/S74L (gray trace) mouse. The traces represent grand averages over all wild-type and mutant rods. In both (A) and (B), the responses of S74L/S74L rods were indistinguishable from those of wild-type rods (see also Table 1).

(C and D) Adaptation-dependent change in amplification constant ($A_{adapted}/A_{dark}$) calculated from the analysis of the a-waves of the ERG responses from wild-type (black) and S74L/S74L (gray) mice. As control experiments, mice were first subjected to ERG recordings (to calculate A_{dark}), then kept in the dark for 10 min instead of being exposed to the adapting light before subjected to ERG recordings again (to calculate $A_{control}$). For each set of experiments (five animals per set), the $A_{control}/A_{dark}$ value shown in (C) represents the average of data collected from test flashes of relative intensities 0.005, 0.01, 0.025, and 0.05 (relative to the brightest flash that saturated the a-wave amplitude). Data in (D) were obtained with a similar experimental protocol, except that the animals were exposed to 10 min steady light of 100 lux or 250 lux and subjected to ERG recordings (to calculate $A_{adapted}$). The $A_{adapted}/A_{dark}$ values obtained by individual test flashes with relative intensities of 0.005, 0.01, 0.025, and 0.05 are shown for the 100 lux light adaptation. Error bars indicate SD. $p < 0.005$ and $p < 0.001$, Student's t test wild-type versus S74L/S74L mice.

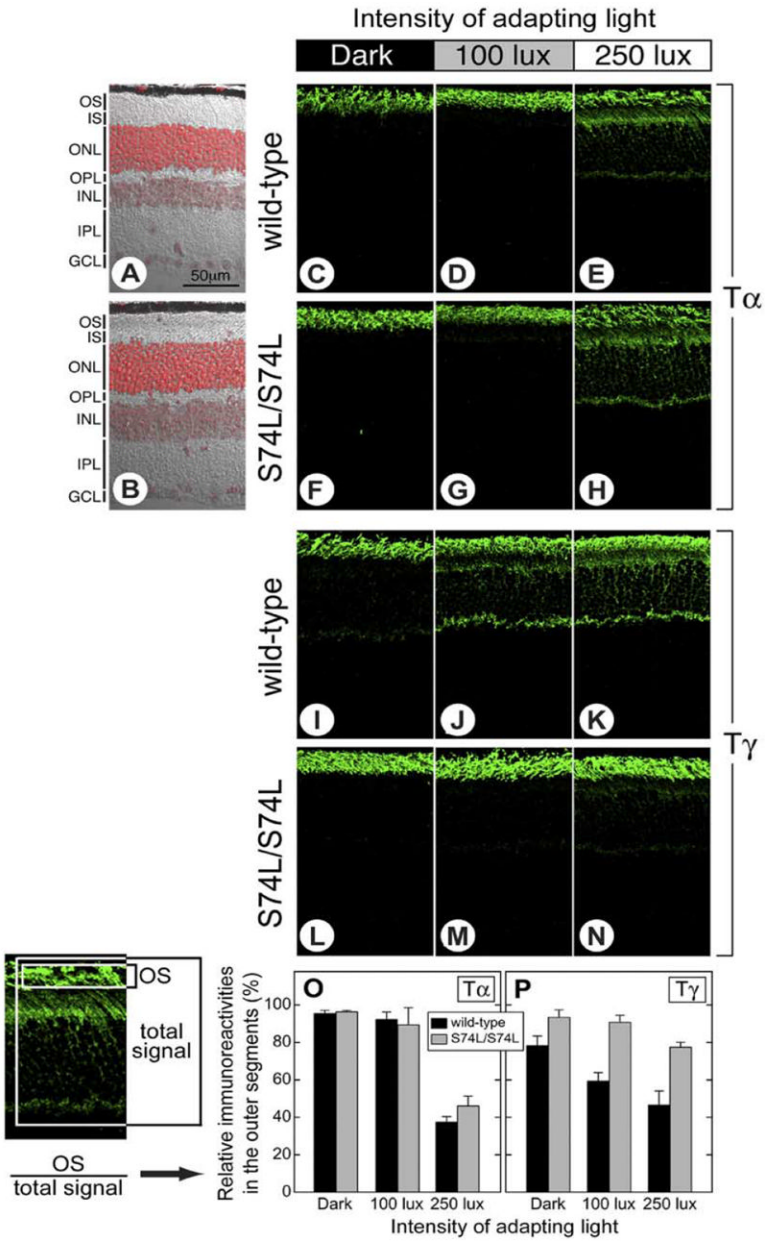

Figure 5. Light-Dependent Translocation of Transducin Subunits in the Retina of S74L Knockin Mouse

(A and B) Differential interference contrast images of the retinal sections from 26-week-old mice of the wild-type (A) and S74L/S74L mutant (B). The image was merged with nuclear staining (red). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. (C–N) Immunohistochemical localization of Tα (C–H) and Tγ (I–N). The sections were prepared from retinas of fully dark-adapted wild-type (C–E and I–K) and S74L/S74L mice (F–H and L–N) before (C, F, I, and L) and after receiving 10 min adapting light of 100 lux (D, G, J, and M) and 250 lux (E, H, K, and N). Incidentally, a longer exposure (~60 min) to the intense light of 250 lux induced translocation of most Tβγ from the rod outer segments even in S74L/S74L mice (data not shown).

(O and P) Transducin content in the outer segments before and after exposure to the adapting light. The immunoreactivities of Tα (O) and Tγ (P) detected in the outer segments and the inner regions of the photoreceptor layer were quantified by Image Gauge software (Fujifilm). The signal intensity of Tα or Tγ in the outer segments was expressed as a percentage of the total intensity of Tα or Tγ in the photoreceptor layer. All values were presented as the mean ± SD of three regions from different sections.

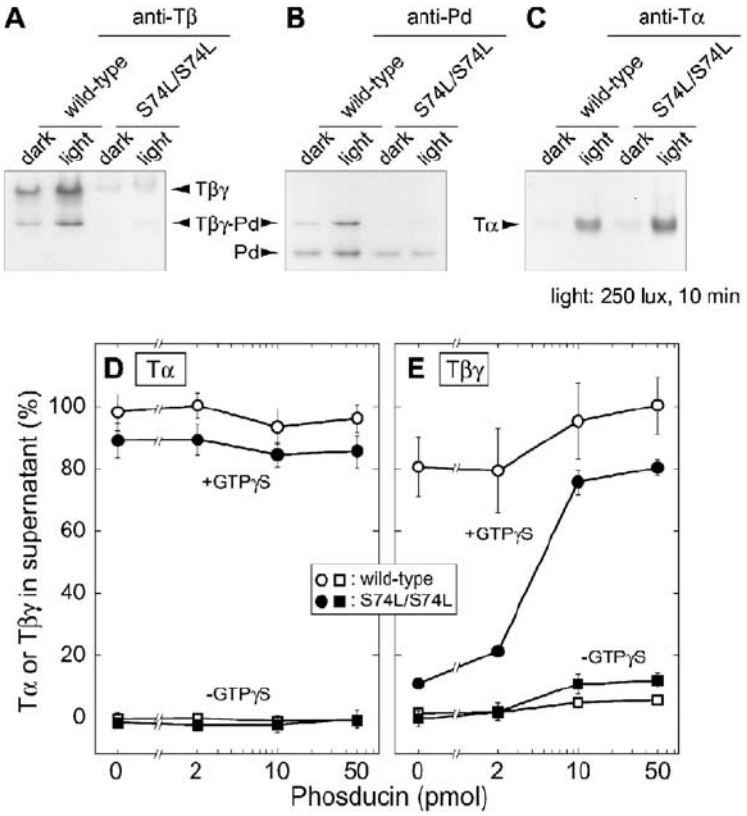

Figure 6. Solubility of Transducin Subunits

(A–C) Light-dependent increase in solubility of transducin subunits. A soluble fraction of the retinal homogenate was prepared from wild-type or S74L/S74L mice after dark or light adaptation (250 lux, 10 min), and it was subjected to native PAGE, followed by immunoblot analysis (0.2 retinas per lane).

(D and E) Effect of the isoprenyl group of Tβγ on solubility of Tα (D) or Tβγ (E) in the presence of various concentrations of exogenously added phosducin. The irradiated outer segment membranes from the wild-type (open symbols) or S74L/S74L mutant (solid) were mixed with recombinant phosducin (0, 2, 10, 50 pmol) in the absence (squares) or presence (circles) of GTPγS (final concentration of 10 μM), and the mixtures were centrifuged to obtain supernatants. The protein levels of Tα and Tβ in the supernatant were quantified by immunoblot analysis. The values were the average ± SEM of results from four independent experiments.

Table 1
Quantitative Measurements of the Flash Responses from Single Rods

	Wild-Type (n = 19)	S74L/S74L (n = 20)
r_{max} (PA)	12.6 ± 2.6	11.6 ± 2.0
i_o (photons/μm^2)	64.3 ± 24.3	57.3 ± 17.9
t_p (ms)	160.0 ± 29.3	172.3 ± 49.3
a (pA)	0.54 ± 0.11	0.52 ± 0.11
t_i (ms)	287.4 ± 152.0	276.1 ± 106.8

r_{max}, the maximal response amplitude; i_o, the flash intensity that gives a half-maximal response; t_p, the time-to-peak of dim-flash response; a, the amplitude of the single-photon response, calculated as the dim-flash response ensemble variance-to-mean ratio; t_i, the integration time defined as the time integral of the dim-flash response divided by its peak amplitude. Dim-flash responses were defined as responses with peak amplitudes less than 0.2 r_{max}. All values were expressed as mean ± SD.