Ubiquitylation of the Transducin $\beta\gamma$ Subunit Complex

REGULATION BY PHOSDUCIN*


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G proteins (Gaβγ) are essential signaling molecules, which dissociate into Ga and Gβγ upon activation by heptahelical membrane receptors. We have identified the $\beta\gamma$ subunit complex of the photoreceptor-specific G protein, transducin (T), as a target of the ubiquitin-proteasome pathway. Ubiquitylated species of the transducin $\gamma$-subunit (Tγ) but not the $\alpha$- or $\beta$-subunits were assembled de novo in bovine photoreceptor preparations. In addition, Tγ was exclusively ubiquitylated when Tβγ was dissociated from Tα. Ubiquitylation of Tβγ on Tγ was selectively catalyzed by human ubiquitin-conjugating enzymes UbcH5 and UbcH7 and was coincident with degradation of the entire Tβγ subunit complex in vitro by a mechanism requiring ATP and the proteasome. We also show that Tβγ association with phosducin, a photoreceptor-specific protein of unknown physiological function, blocks Tβγ ubiquitylation and subsequent degradation. Phosphorylation of phosducin by Ca2+/calmodulin-dependent protein kinase II, which inhibits phosducin-Tβγ complex formation, completely restored Tβγ ubiquitylation and degradation. We conclude that Tβγ is a substrate of the ubiquitin-proteasome pathway and suggest that phosducin serves to protect Tβγ following the light-dependent dissociation of Tαβγ.

Heterotrimeric guanine nucleotide-binding proteins (Gaβγ) constitute a large family of eukaryotic signaling molecules, which transduce signals between seven-helical membrane receptors and intracellular effectors or ion channels (reviewed in Refs. 1–3). Agonist-induced exchange of GDP for GTP on Ga results in dissociation of Ga-GTP from Gβγ, each of which can interact with effectors in different signaling pathways. Hydrolysis of bound GTP promotes reassociation of Ga and Gβγ and termination of the G protein-mediated signal (1–5). Gβγ activity is regulated by at least two classes of additional proteins. They include the G protein-coupled receptor kinases (GRKs) (6) and phosducin (Pd) and phosducin-like proteins (PhLPs) (reviewed in Ref. 7). Phosducin is most highly expressed in the mammalian pineal gland and retina. In the latter it is present in high concentrations (>350 μM) in the cytosol of photoreceptor (i.e. rod) cells (8, 9). Phosducin forms a tight complex with the $\beta$ heterodimer of the photoreceptor-specific G protein, transducin (Taβγ) following light-induced transducin dissociation (10, 11). Formation of the phosducin-Tβγ (Pd-Tβγ) complex reduces the availability of free Tβγ for re-association with Ta-GDP, which is required for subsequent transducin activation. Dark-dependent phosducin phosphorylation by protein kinase A (PKA) or by Ca2+/calmodulin-dependent protein kinase II (CaMKII) reduces phosducin binding to Tβγ by 3- and 300-fold, respectively (11–15). These observations suggested a mechanism in which the cycle of phosducin phosphorylation/dephosphorylation regulates the light sensitivity of the photoreceptor by controlling the amount of transducin available for activation. Such a mechanism might be important for photoreceptor light adaptation, during which potentially saturating levels of light input to photoreceptor cells are countered by desensitization (reviewed in Ref. 16). However, recent studies (9, 17, 18) clearly demonstrate that most phosducin is localized not in the outer segments of photoreceptors, where the light receptor rhodopsin is present and where phototransduction occurs, but in the inner segments, which are primarily responsible for the metabolic functions of the cell. This inconsistency calls for a revision of the proposed role of phosducin in photo

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§§‡‡‡ The abbreviations used are: Gaβγ, guanosine triphosphate-binding protein; Tα, transducin $\alpha$-subunit; Tβγ, transducin $\beta\gamma$ heterodimer; Pd-Tγγ, complex of phosducin and transducin $\beta\gamma$ heterodimer; PdPS73A, mutant phosducin containing a serine–alanine substitution at residue 73; PhLP, phosducin-like protein; GRK, G protein receptor kinase; PKA, CaMP-dependent protein kinase; CaMKII, calcium-calmodulin-dependent protein kinase; UPP, ubiquitin-proteasome pathway; Ub, ubiquitin; E1, ubiquitin-activating enzyme; Ubc, ubiquitin-conjugating enzyme; UbcH, human ubiquitin-conjugating enzyme; E3, ubiquitin isopeptide ligase; Ni-NTA, nickel-nitritriacetic acid; Fl, fraction I (flow through) from ion exchange column; FII, fraction II (high salt eluate) from ion exchange column; MG132, carbobenzoxyl-leucyl-leucyl-leucinal-H; RPE, retinal pigment epithelium; ROS, rod outer segment; endo-LysC, endoproteinase LysC; mAb, monoclonal antibody; DTT, dithiothreitol.

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receptors. A solution might be provided by the observation that, upon continuous illumination, up to 90% of both Tα and Tβγ translocate from the photoreceptor outer segment to the inner segment (Ref. 19 and references therein). However, the functional importance of Tβγ interaction with phosducin in the inner segment is unclear.

The ubiquitin (Ub) proteasome pathway (UPP) provides another potential mechanism of G protein regulation (20, 21). The UPP is a conserved pathway of selective protein modification and degradation that controls levels and activities of many highly regulated eukaryotic proteins (reviewed in Refs. 22, 23). Substrates of the UPP are covalently ligated to one or more monomers of the 8.5 kDa protein, ubiquitin, by the sequential activities of three families of thiol enzymes: Ub-activating enzymes (E1), Ub-conjugating enzymes (Ubc), and Ub-isopeptide ligases (E2) (22, 23). Two E1 enzymes, over 30 Ubc enzymes, and more than 100 E2 ligases have been identified. Selectivity in ubiquitylation is accomplished by the specific interplay between Ubc and E3, which interact with distinct but incompletely understood ubiquitylation signals within substrates. Ubiquitylation signals (also called "degrons" when ubiquitylation targets proteins for degradation) consist of two modules: 1) primary determinants for recognition by Ubc, E3 (and occasionally ancillary factors) and 2) secondary determinants, i.e. one or more lysines to which ubiquitin is covalently attached (reviewed in Ref. 24). Multiple ubiquitin molecules can be subsequently attached to a substrate as an isopeptide-linked polyubiquitin chain. Such chains preferentially target the substrate moiety of the Ub-protein conjugate for degradation by the 26 S proteasome, a multicatalytic, ATP-dependent protease (reviewed in Ref. 25). Although the best recognized function of ubiquitylation is selective targeting of proteins for rapid degradation (22, 23), ubiquitylation per se can regulate protein trafficking, phosphorylation, and other nonproteolytic fates (reviewed in Ref. 26).

The most extensive evidence for the regulation of G protein signaling by the UPP has been obtained in S. cerevisiae, where Ub-dependent proteolysis of Go controls signaling through the mating pheromone receptor (24, 27). We previously identified Tαβγ as a UPP substrate in vitro (20) and proposed that Tαγ was the ubiquitylated subunit, based on detection of a ~30 kDa Ub protein conjugate in the transducin fraction from bovine photoreceptors (28). Interestingly, phosducin has also been suggested to interact with the UPP. Specifically, two-hybrid screens, reconstitution assays and overexpression studies (29, 30) have demonstrated that phosducin (and PhLP) interacts with p45/Sug1, a subunit of the 26 S proteasome 19 S regulatory complex. This observation has led to a suggestion that phosducin and/or PhLP act as adapters linking Gβγ heterodimers to the 26 S proteasome (29–31). Upon delivery to the proteasome, Gβγ could be degraded, or alternatively, refolded by the chaperone activity of the 19 S regulatory complex (32).

Here we report that Tβγ is ubiquitylated on Tγ and that Tγ ubiquitylation targets the Tβγ heterodimer for degradation by the 26 S proteasome in vitro. However, Tβγ is completely resistant to ubiquitylation and degradation when complexed with phosducin. These data provide the first example of Gβγ subunit complexes being UPP substrates and suggest a novel role for phosducin as a protective factor for Tβγ during continuous illumination.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials for electrophoresis were from Bio-Rad laboratories (Hercules, CA). Coomassie Plus protein assay reagent and the Super Signal chemiluminescence detection kit were purchased from Pierce. Na232P was supplied by PerkinElmer Life Sciences. Nickel-nitrilotriacetic acid (Ni-NTA) agarose beads were from Qiagen, Inc. (Valencia, CA). Frozen bovine retinas were purchased from W. L. Lawssch, Co. (Lincoln, NE). Carbobenzoxy-leucyl-leucyl-tyrosine-lysyl-HCL (MG132), ubiquitin aldehyde (Ub-aldehyde), His6-ubiquitin (His6-Ub), rabbit E1, HeLa cell fraction I (FI), bacterially expressed recombinant human Ubc (UbcH) H2, H6, H7, H9, and H10 and active site Cys→Ser mutants of UbcH5c and UbcH7 were purchased from Boston Biochem. Inc (Cambridge, MA). His6-UbcH3 was expressed in Escherichia coli and purified on Ni-NTA agarose. (The His6-UbcH3 plasmid was a generous gift from Dr. Sharon Plon, Texas Children’s Cancer Center, Houston). UbcH5c was expressed in E. coli and purified as previously reported (33). (The UbcH5c plasmid was generously provided by Dr. Simon Wing, Department of Medicine, McGill University). Bacterially expressed bovine UbcH1 (34) was a generous gift from Dr. Cecile Pickart (The Johns Hopkins University, Baltimore, MD). Rabbit reticulocytes were purchased from Pel-Freez Biologicals (Rogers, AR). Unless otherwise specified, all other materials were purchased from Sigma and were the highest grade available.

High speed (85,000 × g) supernatants from rabbit reticulocyte lysate and human retinal pigment epithelial (RPE) cells were prepared as described (20, 28). Ubc-depleted RPE supernatant was prepared by spin dialysis through a Centricon 100 microconcentrator (Millipore, Corp, Bedford, MA). Reticulocyte lystate fraction II (FI1) was prepared as per Herskho and co-workers (35).

**Photoreceptor and Transducin Preparations**—Bovine photoreceptor rod outer segments (ROS) were purified as previously described from dark-adapted retinas on continuous density gradients, and high speed ROS supernatant was obtained (28). A (90/10) mixture of either Tαγ-GTP-S and Tβγ or Tα-AIFβ, and Tβγ was eluted from extensively washed, polymerized ROS membranes in the presence of 100 μM GTP-S (28) or AIFβ (36), respectively. Transducin was depolymerized by digestion with endoproteinase LysC (endo-LysC), which removes Tγ residues 69–71, including the farnesyl moiety at Cys71 (37). Depolymerized Tβγ was purified by sequential chromatography on Cibacron Blue 3GA-agarose and Mono-Q (Amersham Biosciences) (36). Preynlated (nonproteolized) Tβγ was purified on Cibacron Blue CL6B (Dupelco, Inc., Bellefonte, PA) from a mixture of Tα-AIFβ and Tβγ (36). Tβγ purity (absence of Tαγ) was confirmed by immunoblotting. Tβγ activity was confirmed by binding to the C-terminal domain of β-adrenergic receptor kinase (38), with binding assessed by electrophoretic mobility shift on native gels. Tβγ was iodinated to low specific activity (~30,000 cpm/μg) as previously described (20). The Tγ-GTP–S/Tβγ mixture, pure Tβγ and 125I-labeled Tβγ were stored at ~20 °C in 10 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 40% glycerol.

**Phosducin and Phosducin-Tβγ complex**—Pd–Tβγ complex was purified from bovine retinas as previously described (39). Association of Pd and Tβγ was confirmed by conjugation of phosducin and Tβγ on native gels (39). Recombinant rat Pd–MyHCis and Pd toward MyHCis were expressed in E. coli, purified on Ni-NTA resin, buffer-exchanged and stored at ~20 °C in 20 mM Tris (pH 7.5), 100 mM NaCl, 0.5 mM DTT, and 50% glycerol (14, 37, 40). Phosducin–MyHCis–Tβγ complex was formed in vitro by incubating Pd–MyHCis (6 μg) and purified Tβγ (4 μg) in 10–12 μl of 20 mM Tris (pH 7.8), 1 mM DTT for 30 min at 22 °C. The inability of recombinant phosducin to bind Tβγ was confirmed by inhibition of light-induced binding of Tαγ to Tβγ (11). Pd–MyHCis was pentaphosphorylated by CaMKII and purified as described (14). The inability of phosphorylated phosducin to bind to Tβγ was confirmed in Tαγ binding assays (as above).

**Ubiquitylation Assays**—Assays (25 μl) contained 3–12 mg/ml of either ROS or RPE supernatant, reticulocyte lysate, reticulocyte FII, or combined FII and HeLa cell FII, 2 mM ATP, and an ATP-regenerating system, 80 μM MG132, 4 μM Ub-aldehyde, and 200–400 ng/μl Ub or Tβγ (28, 41). Assays also received 2–4 μg of exogenous substrate (i.e. Tαγ-GTP–S/Tβγ, Tγ, or purified or reconstituted Pd–Tβγ complex). To identify Ubcs involved in Tβγ ubiquitylation, some assays were supplemented with 0.2–0.8 μg of individual recombinant human Ubc or 4 μg of active site Cys→Ser mutants of UbcH5c or UbcH7. To control for differences in Ubc activity, the amount of each Ubc added to assays varied as the inverse of Ubc activity, which was quantitated by autoradiography as the formation of 125I-labeled Ub–Ubc thiol esters in the presence of purified rabbit E1 and ATP (41). Phosducin phosphorylation was prevented by inclusion of the kinase inhibitor H-89 (Calbiochem, Novabiochem, San Diego, CA) at a final concentration (400 μM) sufficient to inhibit both PKA (K0.25 μM) and CaMKII (K0.30 μM). Phosducin phosphorylation was stabilized against degradation by inclusion of microcin LT (10 μM final concentration).

Ubiquitylation assays were incubated at 37 °C for the times indicated.
cated and were terminated by boiling with gel loading buffer containing 2-mercaptoethanol. Ubiquitylated proteins were visualized by Western blotting (41). Primary antibodies included the following: polyclonal IgGs raised against peptide sequences of mammalian Goα1 (sc-389), Gβ1 (sc-379), and Gγ1 (sc-373) (all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal antibody (mAb) TF-15 (45), and rabbit antiserum raised against the LAP-636 peptide of bovine Gβ1 (44) (all generously provided by Dr. Bernard Fung, University of California at Los Angeles), polyclonal IgG (BN-1) raised against a conserved peptide sequence of bovine Gβ1 (42), mAb TF-28 raised against bovine Gγ1 (45), and rabbit antiserum raised against the LAP-636 peptide of bovine Gβ1 (44) (all generously provided by Dr. Mel Simon, California Institute of Technology, Pasadena, CA), rabbit serum (“Gertie”) (13), which recognizes phosducin and PhLP, anti-phosducin mAb 1D6 (a generous gift of Dr. Lawrence Donoso, Wills Eye Hospital, Philadelphia, PA), polyclonal IgG, which recognizes free and conjugated ubiquitin (28) or appropriate preimmune or nonimmune IgG/serum. Specific binding was detected with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and visualized by ECL. Signal intensities were quantified by digital densitometry (Amersham Biosciences), using standard curves of antigens (in assay mixtures) to determine the linear range of ECL signal detection.

**Ni-NTA-agarose Isolation of Ty-His6-Ub Species Synthesized De Novo**—Ubiquitylation assays (70 µl) were incubated for 1 h and terminated with Buffer I (8 mM urea, 0.1% Tween 20, 20 mM 2-mercaptoethanol, 0.1 mM imidazole, pH 8.0, 10 mM Tris-HCl, pH 8.0). Next, 25 µl of washed Ni-NTA beads (50% slurry) were added and incubated (30 min, 22 °C) with gentle rotation. The beads were subjected to 5 cycles of washing with Buffer II (Buffer I, pH 6.5). His6-Ub-protein conjugates were then eluted with Buffer III (Buffer I, pH 4.5 containing 0.5 M imidazole), boiled in reducing gel buffer, and analyzed by Western blotting.

**Proteolysis Assay**—Proteolysis assays were constituted similarly to ubiquitylation assays (above), except that Ub-aldehyde was omitted, and reactions were conducted in the presence and absence of both ATP and MG132. Degradation (i.e. loss) of specific proteins was assessed by densitometry of immobiloblot assay mixtures or, when 125I-labeled Tβγ was used as a substrate, by γ-counting of acid-precipitable cpm (20, 29).

**Fig. 1.** De novo ubiquitylation of Tβ by the photoreceptor UPP. A. ATP-dependent synthesis of higher mass species of Tβ in vitro. Ubiquitin-supplemented supernatant from gradient-purified photoreceptor (rod) outer segments was incubated in the presence (lane 2) or absence (lane 1) of ATP (see “Experimental Procedures”). Western blots of reaction mixtures were probed with IgGs directed against Ta (upper panel), Tβ (middle panel), and Tγ (lower panel). Molecular mass markers are at left. A representative assay of three is shown. B. Isolation of His6-ubiquitylated Tβ on nickel agarose. ATP-supplemented photoreceptor ubiquitylation assays (as in A) were supplemented with either native Ub (lanes 2, 4, and 6) or His6-Ub (lanes 1, 3, and 5). Reaction mixtures were incubated with Ni-NTA beads under denaturing conditions, and proteins bound to pelletted beads were subjected to SDS-PAGE and Western blotting with anti-Ta IgG. Lanes 1 and 2 contain ubiquitylation assay supernatants; lanes 3 and 4 contain cleared ubiquitylation assay supernatants following incubation with andpelleting of Ni-NTA beads; lanes 5 and 6 contain proteins pelletted with Ni-NTA beads. Lanes 5 and 6 contain 10-fold more input than lanes 1–4. The slower migrating mass species of Ty-His6-Ub in lane 4 is bleed over from lane 5. Ty-Ub3 is so designated based on apparent molecular mass (30 kDa), but it may contain only two ubiquitins (see “Results”). Molecular mass markers are at left. A representative assay of two is shown.

**RESULTS**

**Identification of Transducin Subunits That Are Ubiquitylated in Vitro**—To identify subunits of transducin that are ubiquitylated in vitro, ubiquitylation assays were conducted with supernatant from gradient-purified, dark-adapted bovine ROS. This preparation contains ubiquitin-conjugating enzyme activities and endogenous, heterotrimeric transducin (Taβγ) (28). Proteins were immunoblotted and probed with antibodies raised against Ta, Tβ, and Tγ. No higher mass (i.e. ubiquitylated) forms of Ta or Tβ were detected in reaction mixtures supplemented with ATP and Ub (Fig. 1A, lane 2, upper and middle panels, respectively). However, higher mass species of Tγ (Ty15K, Ty30K) were detected, and their formation was ATP-dependent, consistent with the requirement of ATP for ubiquitylation (Fig. 1A, lower panel; compare lanes 1 and 2).

The apparent molecular mass of Tγ is ~7 kDa, allowing us to rationalize these higher mass forms of Tγ as Ty-Ub3 (15 kDa) and Ty-Ub3 (30 kDa), respectively. However, it is plausible that the 30 kDa ubiquitylated species contains only two ubiquitins (Ty-Ub3), but migrates anomalously (at 30 kDa rather than 22 kDa) in SDS-PAGE gels. An identical mass distribution for Tγ staining was obtained with additional anti-Tγ antibodies (data not shown). These results suggest that Tγβγ was ubiquitylated exclusively on Tγ, with up to three ubiquitin moieties incorporated.

Ty ubiquitylation by the photoreceptor UPP was confirmed by supplementing ROS preparations with His6-Ub, isolating His6-ubiquitylated species on Ni-NTA beads under denaturing...
Tyndalinububutination assays with purified T/H9252/H9253/H9251
conclude that T/H9252/H9253/H9251 blotting (Fig. 1B, compare lanes 1 and 2); 2) cleavage of TyUb1 and TyUb2 bands from His8-Ub-supplemented assays by Ni-NTA beads (Fig. 1B, compare lanes 1 and 3); 3) enrichment for TyUb1 and TyUb2 in Ni-NTA pellets from assays containing His8-Ub (Fig. 1B, lane 5) but not from assays supplemented with native Ub (Fig. 1B, lane 6); and 4) ubiquitin immunoreactivity of 15- and 30-kDa species on Western blots of Ni-NTA pellets from assays containing His8-Ub (data not shown). No Ta or Tβ immunoreactivity was detected on Western blots of Ni-NTA pellets (data not shown); note that the Tβγ complex was dissociated under the denaturing conditions of these pull-down assays). In summary, these data demonstrate that the higher molecular mass forms of Ty were ubiquitylated in Ty species and support the notion that transducin ubiquitylation occurred exclusively on Ty.

To Is Not Required for Tβy Ubiquitylation—The data from Fig. 1 indicate that Ty can be ubiquitylated as a part of the soluble Tαγtrimer. We next addressed if Ty could also be ubiquitylated following dissociation of Tβγ from Tα. We incubated a mixture of light-dissociated transducin subunits (Tc-GTP·S/Tβγ) in RPE supernatant, a cell-free preparation used as the source of UPP enzymes (20). The assays were supplemented with His8-Ub and ATP, as well as with MG132 and Ub-aldehyde, inhibitors of the proteasome and deubiquitylating enzymes, respectively. His8-Ub-protein conjugates were then isolated under denaturing conditions with Ni-NTA agarose beads, and proteins in the supernatants and those attached to the precipitated beads were analyzed by Western blotting for the presence of Ty. A trace of TyUb1 was detected in reaction mixtures containing transducin but no exogenous ATP (Fig. 2A, lane 2), but this trace amount was insufficient for detectable isolation (Fig. 2A, lane 5). In contrast, significant levels of TyUb1 and some TyUb2 were detected in the ATP-supplemented samples (Fig. 2A, lane 3). In this case, TyUb1 was readily detected in Ni-NTA precipitates of these reactions (Fig. 2A, lane 6). These data indicate that Ty was ligated to His8-Ub through an ATP-dependent mechanism. Moreover, since Tc-GTP·S and Tβγ were dissociated in these assays, we conclude that Tα is not required for the ubiquitylation of Ty within the Tβγ subunit complex.

We further supported this conclusion by conducting ubiquitylation assays with purified Tβγ. These experiments used reticulocyte lysate, a classical cell-free UPP system (35), which was supplemented with ATP in the presence or absence of MG132 and Ub-aldehyde. Western blotting confirmed the synthesis of TyUb1, which was detectable in assays containing MG132 and Ub-aldehyde (Fig. 2B, compare lanes 1 and 2). Consistent with results obtained with the Gα-interaction Tβγ mixture, ubiquitylation of purified Tβγ occurred selectively on Ty, since no higher molecular mass species of Tβγ were detectable on Western blots of ATP-supplemented reactions (data not shown).

Tβγ Is a Substrate for Ub-dependent Proteolysis—In the experiments illustrated in Fig. 2B, we also noted that TyUb1 was only detectable in the presence of MG132 and Ub-aldehyde (Fig. 2B, compare lanes 2 and 4). This observation suggested that ubiquitylated Tβγ was a substrate for either the 26 S proteasome and/or de-ubiquitylating enzymes. To confirm this possibility, we conducted UPP proteolysis assays using 125I-labeled Tβγ as substrate. The hallmarks of ubiquitin-dependent proteolysis are the requirements for ATP and proteasome activity. We therefore monitored the release of acid-soluble radioactivity when 125I-labeled Tβγ was incubated in reticulocyte lysate in the presence or absence of ATP and MG132. The data (Fig. 3A) indicate that ~13% of Tβγ was degraded within 30 min in the presence of ATP, whereas less than 2% was degraded in the absence of ATP. In addition, ATP-dependent proteolysis of 125I-labeled Tβγ was substantially blocked by MG132 (Fig. 3A). These data suggest that 125I-labeled Tβγ was degraded almost exclusively by the UPP.

Autoradiography of 125I-labeled Tβγ degradation assays following 2 h of incubation in reticulocyte lysate (as in Fig. 3A) reveals that levels of both Tβγ and Ty were reduced in the presence of ATP (Fig. 3B, compare lanes 1 and 2) and were stabilized in the presence of MG132 (Fig. 3B, compare lanes 2 and 3). These results suggest that, despite the fact that only Ty becomes ubiquitylated, the entire Tβγ complex is subsequently degraded. To verify that the native (noniodinated) Tβγ complex was also degraded by the UPP, we incubated non-radiolabeled Tβγ in ATP-supplemented reticulocyte lysate and assessed the levels of Tβγ and Ty, which remained after 90 min by Western blotting (Fig. 3C). The levels of both Tβγ and Ty were reduced (~40–60%) following incubation in ATP-supplemented assays (Fig. 3C, compare lanes 1 and 2 and 3 and 4). Data obtained with radiolabeled and native Tβγ therefore support the conclusion that both subunits of the Tβγ complex are degraded by the UPP.

Identification of Ubiquitin-conjugating Enzymes That Catalyze Tβγ Ubiquitylation and Promote Tβγ Degradation—We then identified Ubc species that can catalyze Ty ubiquitylation. In an initial screen using Tβγ as substrate and RPE supernatant as the UPP source, we found that Ty ubiquitylation could...
be enhanced by supplementation of ubiquitylation assays with recombinant human UbcH5a, UbcH5c, or UbcH7 but not with UbcH1, UbcH2, UbcH3, UbcH6, UbcH9, or UbcH10 (50). We then employed an established approach (35, 46) to evaluate the requirement of UbcH5/UbcH7 in Tβ degradation. In this approach, rabbit reticulocyte lysate was subjected to DEAE chromatography, and UbcH5 and UbcH7 were removed in the column flow-through (F1). The high salt eluate (FII) contains E1, other Ubc, and E3. Although some Tβ moved in the column flow-through (FII), levels of both 125I-labeled Tβ and 125I-labeled Tγ were reduced in the presence of ATP (compare lanes 1 and 2) but were enhanced when ATP-supplemented assays contained MG132 (compare lanes 2 and 3). Note that Tγ consistently incorporates dramatically less radiolabel as compared with Tβ. C, degradation of native Tβγ. Native Tβγ was incubated in reticulocyte lysate in the presence of ATP. Levels of Tβ (upper panel) and Tγ (lower panel) were assessed after 90 min (T90, lanes 2 and 4) and after 90 min (T90, lanes 2 and 4) in duplicate assays (I, II) by Western blotting with anti-Tβ and anti-Tγ IgGs. A representative experiment of three is shown.

For degradation, we assessed the effect of exogenous UbcH5c on the release of acid-soluble radiolabel from 125I-labeled Tβγ (20, 28). 125I-labeled Tβγ was incubated in ATP- and Ub-supplemented RPE supernatant that had been previously depleted of Ubes by dialysis (see “Experimental Procedures”). The addition of UbcH5c to Ubc-depleted RPE supernatant was associated with a 3-fold increase in the magnitude of ATP/Ub-dependent degradation of 125I-labeled Tβγ (p < 0.001; Fig. 4D). In addition, whereas less than 40% of 125I-labeled Tβγ was degraded in 90 min by reticulocyte lysate (as in Fig. 3, A and B), less than 5% of 125I-labeled Tβγ was degraded during the same period by reticulocyte FII, which is depleted of UbcH5 isofoms (n = 2 assays in duplicate). Together, these data support a role for UbcH5 in the degradation of 125I-labeled Tβγ.

Mammalian UbcH5s and their yeast homologues are implicated in the selective ubiquitylation and degradation of abnormal and damaged proteins (22, 47). To rule out the possibility that UbcH5-dependent degradation of 125I-labeled Tβγ resulted from Tβγ iodonation (i.e. oxidation), we also assessed UbcH5-mediated degradation of native (noniodinated) Tβγ. Tβγ was incubated in ATP- and Ub-supplemented reticulocyte FII in the presence and absence of exogenous UbcH5c. After 90 min, levels of Tβ were assessed by Western blotting (Fig. 4E, left panel) and densitometry (Fig. 4E, middle and right panels). Reaction mixtures supplemented with UbcH5c contained on average 40% less Tβ than reaction mixtures lacking UbcH5c (p < 0.02) (Fig. 4E, compare lanes 1 and 2 and 3 and 4). These results confirm that UbcH5 promotes the degradation of Tβγ in vitro. Considered together, ubiquitylation data (Fig. 4, A–C) and proteolysis data (Fig. 4, D and E) strongly suggest that ubiquitylation of Tγ by UbcH5 destabilizes the Tβγ complex, presumably by targeting Tβγ to the 26 S proteasome (Fig. 3, A and B).

Phosducin Binding to Tβγ Prevents Tβγ Ubiquitylation—Phosducin binds Tβγ with high affinity, covering portions of Tβ (37, 48, 49) and putatively inducing conformational changes in the Tγ C terminus (49). To investigate the potential effects of

3 M. Ohin and X. Gong, unpublished data.
phosducin binding on Tβγ ubiquitylation, we conducted two similar groups of experiments. In the first, we purified the phosducin-Tβγ complex (Pd-Tβγ) from bovine retina and compared its ubiquitylation with ubiquitylation of purified Tβγ. As demonstrated above (Fig. 4A), when Tβγ was incubated in Ubch5-supplemented FII, Tγ was ubiquitylated in an ATP-dependent manner (Fig. 5A, left panel; compare lanes 1 and 2). In contrast, Tγ was not ubiquitylated when the Pd-Tβγ complex was incubated in Ubch5-supplemented FII (Fig. 5A, left panel; compare lanes 2 and 3). The possibilities that Tβ or phosducin were ubiquitylated in these experiments were also investigated, but Western blotting failed to detect higher mass species of either protein (Fig. 5A, middle and right panels). Thus, in contrast to Tβγ, the Pd-Tβγ complex does not appear to be a substrate for de novo ubiquitylation.

In the second group of experiments, we reconstituted the Pd-Tβγ complex in vitro and then compared ubiquitylation of the reconstituted complex with ubiquitylation of native Tβγ. In

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**Fig. 4. Identification of the Ubc that catalyzes Tγ ubiquitylation and promotes Tβγ degradation.** A, HeLa cell F1, Ubch5, or Ubch7 enhance Tβγ ubiquitylation in reticulocyte Fraction II. Reticulocyte Fraction II, which contains diminished levels of Ubch5 and Ubch7 orthologues, was incubated with Tβγ in the presence of ATP, His6-Ub, MG132, and Ub-aldehyde. Some incubations also received either HeLa cell Fraction I (F1, lane 2), which is enriched for Ubch5 isoforms and Ubch7, recombinant Ubch5c (lane 3), or recombinant Ubch7 (lane 4) (each at 70 nm final concentration). Ubiquitylation reactions were terminated after 30 min, and proteins were subjected to Western blotting with anti-Tβγ, nonspecific band. Representative data are from one of three assays. B, an active site Cys→Ser mutant (m) Ubch5, but not mutant Ubch7 inhibits Tβγ ubiquitylation. Tβγ ubiquitylation assays were conducted in His6-Ub-supplemented reticulocyte lysate in the absence (lane 1) or presence (lanes 2–4) of ATP. Some assay mixtures were preincubated with mUbch5 (lane 3) or mUbch7 (lane 4) (each at a final concentration of 0.75 μM). Western blots were probed with anti-Tγ IgG. A representative assay of two is shown. C, the Tβ subunit is not ubiquitylated in the presence of HeLa cell F1, Ubch5, or Ubch7. Ubiquitylation reactions (as in A) were supplemented with HeLa cell F1 (lane 1), Ubch5c (lane 2), or Ubch7 (lane 3). Western blots of assay mixtures were probed with anti-Tβγ, serum (LAP636). LAP636 cross-reactivity was assessed in the absence (lane 4) or presence (lanes 2–4) of exogenous Ubch5c (as in A). Left panel, levels of Tβ remaining after 90 min were assessed on Western blots probed with anti-Tβγ IgG. One of two experiments performed with duplicate Tβγ incubations (I, II) is shown. Middle panel, densitometry data from an experiment (as in left panel) performed in triplicate. *, p < 0.05 for effect of Ubch5. Right panel, Tβγ standard curve confirming that densitometry data plotted in the middle panel were within the linear range of ECL detection (r² = 0.996).
these experiments we used the phosphorylation-resistant phosducin mutant, PdS73A (see “Experimental Procedures” for details). As expected, native Tβγ was ubiquitylated by RPE supernatant in an ATP-dependent manner, generating TγUb1 and TγUb3 (Fig. 5B, compare lanes 1 and 2). In contrast, Tβγ which had been preincubated with PdS73A was not ubiquitylated (Fig. 5B, compare lanes 2 and 3). Consistent with results obtained with the Pd-Tβγ complex purified from bovine retina (Fig. 5A, middle and right panels), we detected no Ub protein conjugates of Tβ or phosducin (data not shown). Importantly, the ability of PdS73A to inhibit ubiquitylation in these assays appears specific for Tβγ, as the extent and pattern of global protein ubiquitylation was similar in the presence or absence of PdS73A (Fig. 5C, compare lanes 2 and 3). These results confirm that the binding of phosducin to Tβγ inhibits Tγ ubiquitylation and does not promote ubiquitylation of either Tγ or phosducin.

**Phosphorylated Phosducin Fails to Protect Tβγ from Ubiquitylation**—Phosphorylation of phosducin by CaMKII, which is thought to occur in the dark, reduces the affinity of phosducin for Tβγ by ∼300-fold (14). To assess potential effects of phosphorylation on phosducin’s ability to inhibit Tβγ ubiquitylation, we compared the ability of PdS73A and CaMKII-phosphorylated phosducin (Pd−p) to inhibit Tβγ ubiquitylation. As shown in Fig. 5D, in the absence of exogenous PdS73A, TγUb1 and TγUb3 were formed in an ATP-dependent manner (compare lanes 2 and 3). As expected, preincubation of Tβγ with PdS73A abrogated formation of TγUb1 and TγUb3 (Fig. 5D, compare lanes 3 and 4). However, preincubation of Tβγ with CaMKII-phosphorylated phosducin had no significant effect on de novo formation of TγUb1 or TγUb3 (Fig. 5D, compare lane 5 with lanes 3 and 2). Thus, in contrast to PdS73A, phosphorylated phosducin was permissive for Tβγ ubiquitylation. Confirmation that PdS73A and CaMKII-phosphorylated phosducin remained differentially phosphorylated throughout the duration of the ubiquitylation assay is shown by the retarded electrophoretic migration of CaMKII-phosphorylated phosducin on SDS-PAGE gels (Fig. 5D, right panel).

**Effects of Phosducin Binding on Tβγ Proteolysis by the UPP**—Ubiquitylation targets Tβγ for degradation by the UPP (Figs. 2B, 3, and 4, D and E). Based on the ability of phosducin to block Tβγ ubiquitylation (above), we predicted that formation of the Pd-Tβ complex would protect Tβγ fromUb-dependent degradation. Indeed, when purified, retina-derived Pd-Tβγ complex was incubated in ATP-supplemented reticulocyte lysate, we observed no loss of either Tβ (not shown) or Tγ (Fig. 6A, compare lanes 1 and 2 and 3 and 4). We also reconstituted the PdS73A-Tβγ complex in vitro (as above) and added reticulocyte FII, which had been supplemented with Ub, ATP, and UbHsc (as in Fig. 4E). Levels of Tβ and Tγ remaining after 90 min were visualized by Western blotting. Preincubation of Tβγ with PdS73A was consistently associated with the retention of more immunoreactive Tβ and Tγ as compared with assays lacking PdS73A (Fig. 6B, compare lanes 1 and 2, 3 and 4, 5 and 6). These data suggest that formation of the Pd-Tβγ complex stabilizes Tβγ.

To confirm that phosducin binding inhibits the proteolytic degradation of Tβγ, we preincubated 125I-labeled Tβγ in the presence or absence of PdS73A and compared the ATP/Ub-dependent release of acid-soluble counts per min (20). Preincubation with PdS73A consistently blocked ≥50% of the ATP/Ub-dependent release of acid-soluble cpm from 125I-labeled Tβγ (p = 0.001) (Fig. 6C). We conclude that phosducin binding stabilizes Tβγ against in vitro degradation by the UPP, consistent with the ability of phosducin to abrogate Tβγ ubiquitylation.

**Phosducin Does Not Co-purify with the 26 S Proteasome or Its p54/Sug1 Subunit**—The inhibitory effect of phosducin on Tβγ proteolysis might seem counterintuitive, given the proposed role for phosducin and PhLP as adapter proteins linking Tβγ to the 26 S proteasome through the p45/Sug1 proteasome subunit (29–31). To assess potential interaction of phosducin (and PhLP) with the 26 S proteasome, we fractionated supernatant...
Aging the presence of 26 S proteasomes in these fractions (Fig. 7, lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of Pd-TβγA and then incubated for 90 min with reticulocyte FII, which was supplemented with ATP, Ub, and UbcH5c (as in Fig. 4). Levels of Ty and Tβ remaining were visualized by Western blotting. A representative assay of two performed in triplicate (I, II, III) is shown.

C.

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\begin{array}{c|c|c}
\text{Gradient Fraction} & \text{Density} & \\
\hline
1 & 33 & \\
2 & 75 & \\
3 & 75 & \\
4 & 75 & \\
5 & 75 & \\
6 & 75 & \\
7 & 75 & \\
8 & 75 & \\
9 & 75 & \\
10 & 75 & \\
11 & 75 & \\
\end{array}
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**DISCUSSION**

During the visual photoreponse, Tβγ dissociates from Tα, and up to 90% of the translocates from the photoreceptor outer segment to the inner segment, where they reside until illumination diminishes (Ref. 19 and references therein). The inner segment is rich in 26 S proteasomes and phosducin, both implicated in interacting with Tβγ (7–15, 18, 28–31). The goals of this study were to determine whether Tβγ is a substrate of the UPP and to address whether phosducin binding to Tβγ modulated Tβγ processing by this pathway. Our two central observations are that Tβγ is a UPP substrate and that binding of phosducin to Tβγ blocks both Tβγ ubiquitylation and its subsequent degradation. These observations suggest that phosducin may function to stabilize Tβγ during the light phase of the normal diurnal cycle.

**Tβγ Ubiquitylation and Degradation**—The present work provides the first direct evidence that a G protein βγ subunit complex can be selectively targeted by the UPP. We have found that Tβγ in vertebrate rods is specifically ubiquitylated on Ty and that ubiquitylation of Ty marks Tβγ for proteolysis by the 26 S proteasome in heterologous cell free systems. We also found that two central observations are that Tβγ is a UPP substrate and that binding of phosducin to Tβγ blocks both Tβγ ubiquitylation and its subsequent degradation. These observations suggest that phosducin may function to stabilize Tβγ during the light phase of the normal diurnal cycle.

**PHOSDUCIN BINDING BLOCKS DEGRADATION OF Tβγ BY THE UPP.** A, stability of Ty in the Pd-Tβγ complex. Pd-Tβγ complex was purified from bovine retina and incubated in ATP-supplemented reticulocyte lysate. Levels of Ty were assessed on Western blots at the start of reactions (T0) and after 90 min (T90). A representative assay of two performed in duplicate (I, II) is shown. B, Tβ and Ty are stabilized in the reconstituted Pd-Tβγ complex. Tβγ was preincubated in the presence (lanes 1, 3, and 5) or absence (lanes 2, 4, and 6) of Pd-TβγA and then incubated for 90 min with reticulocyte FII, which was supplemented with ATP, Ub, and UbcH5c (as in Fig. 4). Levels of Ty and Tβ remaining were visualized by Western blotting. A representative assay of two performed in triplicate (I, II, III) is shown. C, ATP/Ub-dependent degradation of 125I-labeled Tβγ is inhibited by phosducin. 125I-labeled Tβγ was preincubated in the presence (+) or absence (−) of Pd-TβγA and then incubated for 30 min with RPE supernatant in the presence or absence of ATP and Ub. ATP/Ub-dependent proteolysis was determined from acid-soluble cpm and used to calculate the percent inhibition of ATP/Ub-dependent proteolysis due to phosducin. Proteolysis in the absence of phosducin averaged ~8%. Data are from three experiments conducted in duplicate. ***, p = 0.001 for effect of phosducin.

**RETINAL PHOSDUCIN IS NOT ASSOCIATED WITH P45/SUG1.** ATP-supplemented lysates of light-adapted bovine retinas were fractionated on glycerol sedimentation gradients. Gradient fractions were Western blotted with antibodies recognizing a common 32-kDa subunit of the 20 S proteasome core particle, p45/Sug1, phosducin, and PhLP under conditions (light adaptation) that promote the formation of the Pd-Tβγ complex. This conclusion is more consistent with the position of PhLP. Molecular mass markers are at left. A representative experiment of three is shown.

**FIG. 6.** 

**Transducin βγ Ubiquitylation, Inhibition by Phosducin**

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present evidence that UbcH5 and UbcH7, which are ubiquitously expressed in mammalian tissues, can catalyze Tβγ ubiquitination in vitro. The ability of both recombinant UbcH5 and UbcH7 to promote the ubiquitination of Tβγ is consistent with previous experiments demonstrating substrate overlap between these two Ubc proteins (51, 52). However, the failure of a dominant negative UbcH7 to inhibit Tβγ ubiquitination argues against an essential role for UbcH7 in Tβγ ubiquitination, although our data do not exclude the possibility that UbcH7 participates with UbcH5 in a combinatorial mechanism (53) by which Tβγ is ubiquitinated in vivo. Three identified UbcH5 isoforms (a, b, and c) are >90% identical (54), but nonetheless exhibit subtle differences in substrate specificity (55). The UbcH5 isoforms in mammalian photoreceptor cells remain to be identified.

It is generally accepted that extensive polyubiquitination is required for substrates to be efficiently recognized and degraded by the 26 S proteasome (reviewed in Ref. 56). Our observations of relatively robust Tβγ degradation may therefore appear surprising, given our detection of Tγ species containing not more than four ubiquitin moieties, with mono-ubiquitylated species predominating. At least one prior study (57) reported that a Ub protein conjugate bearing a single ubiquitin was a competent substrate for degradation by the 26 S proteasome, with the rate of proteolysis enhanced by the addition of each additional ubiquitin moiety. Alternatively, the failure to observe ubiquitylated species of Tγ containing more than four ubiquitins could reflect the fact that such Tγ conjugates are highly unstable (due to disassembly or degradation) and never accumulated to detectable levels. Both observations support the conclusion that the extent of Tγ ubiquitination in the present study was sufficient to target the Tβγ heterodimer to the proteasome. Thus, the ubiquitylation signal on Tβγ comprises a degradation signal or degron.

The Organization of the Tβγ Degron—Our data suggest several important features of the Tβγ degron. First, the degron is not altered by the presence of Ta, since essentially identical ubiquitination patterns were observed with Taβγ (Fig. 1) and Tβγ (Fig. 2). Second, the farnesylated C terminus of Tγ is not an essential component of the degron, since the pattern of Tγ ubiquitination and the ability of phosducin to inhibit this ubiquitination were comparable when either farnesylated Tγ containing more than four ubiquitins could reflect the fact that such Tγ conjugates are highly unstable (due to disassembly or degradation) and never accumulated to detectable levels. Both observations support the conclusion that the extent of Tγ ubiquitination in the present study was sufficient to target the Tβγ heterodimer to the proteasome. Thus, the ubiquitylation signal on Tβγ comprises a degradation signal or degron.

The Role of Phosducin in Transducin Stabilization—We have demonstrated that phosducin is a negative regulator of Tβγ ubiquitination and degradation. We therefore propose an in vivo function for phosducin as a stabilizer of Tβγ following the translocation of Tβγ from rod outer segments to the inner segments under sustained illumination. We suggest that the following sequence of events occurs during the normal diurnal cycle. In the light, transducin translocates to the inner segment, where Tβγ binds to phosducin. This binding protects Tβγ from ubiquitination and degradation by the UPP. Formation of the Pd-Tβγ complex may protect Ta as well, since phosducin hinders Ta from binding to Tβγ (11, 37), and the Tαβγ trimer can be ubiquitinated on Tγ (Fig. 1) and degraded by the UPP (20, 21). With onset of darkness, phosducin is phosphorylated at up to five serine residues (14), which reduces phosducin affinity for Tβγ. This allows Tβγ to return to the outer segment, where the absence of proteasomes ensures Tβγ (and Tαβγ) stability. Consistent with the proposed role of phosducin in Tβγ stability, the retinas of Ta knockout mice, which contain only free Tβγ, also contain increased levels of phosducin (59), perhaps as an adaptive response for Tβγ stabilization.

In addition to controlling levels of highly regulated proteins, the UPP removes aberrant (and thus potentially toxic) proteins from cells (21, 47). In this regard, the formation of the Pd-Tβγ complex may function in Tβγ quality control to the extent that damaged Tβγ heterodimers fail to associate normally with phosducin in the photoreceptor inner segment. In the absence of tight phosducin binding, Tβγ would display unmasked ubiquitylation signals for recognition by the UPP. Degron unmasking is proposed as a general mechanism by which damaged multimeric proteins are targeted for degradation (60). The selective removal of functionally altered transducin subunits may be vital to visual health, as dysregulated phototransduction signaling can induce photoreceptor cell apoptosis and retinal degeneration (reviewed in Ref. 61).

In conclusion, we note that our data do not rule out nonproteolytic functions of Tβγ ubiquitination. Monoubiquitination, which is known to promote cytosolic trafficking of membrane proteins (reviewed in Ref. 26), could facilitate the light-dependent translocation of the lipophylic Tβγ subunit complex between photoreceptor outer and inner segments.

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