Phosducin-like Protein Regulates G-Protein βγ Folding by Interaction with Tailless Complex Polypeptide-1α

DEPHOSPHORYLATION OR SPlicing OF PhLP TURNS THE SWITCH TOWARD REGULATION OF Gβγ FOLDING

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Phosducin-like protein (PhLP) exists in two splice variants PhLP<sub>LONG</sub> (PhLP<sub>L</sub>) and PhLP<sub>SHORT</sub> (PhLP<sub>S</sub>). Whereas PhLP<sub>L</sub> directly inhibits Gβγ-stimulated signaling, the Gβγ-inhibitory mechanism of PhLP<sub>S</sub> is not understood. We report here that inhibition of Gβγ signaling in intact HEK cells by PhLP<sub>S</sub> was independent of direct Gβγ binding; however, PhLP<sub>S</sub> caused down-regulation of Gβ and Gγ proteins. The down-regulation was partially suppressed by lactacystine, indicating the involvement of proteasomal degradation. N-terminal fusion of Gβ or Gγ with a dye-labeling protein resulted in their stabilization against down-regulation by PhLP<sub>S</sub> but did not lead to a functional rescue. Moreover, in the presence of PhLP<sub>S</sub>, stabilized Gγ subunits did not coprecipitate with stabilized Gβ subunits, suggesting that PhLP<sub>S</sub> might interfere with Gβγ folding. PhLP<sub>S</sub> and several truncated mutants of PhLP<sub>S</sub> interacted with the subunit talless complex polypeptide-1α (TCP-1α) of the CCT chaperonin complex, which is involved in protein folding. Knock-down of TCP-1α in HEK cells by small interfering RNA also led to down-regulation of Gβγ. We therefore conclude that the strong inhibitory action of PhLP<sub>S</sub> on Gβγ signaling is the result of a previously unrecognized mechanism of Gβγ-regulation, inhibition of Gβγ-folding by interference with TCP-1α.

The two splice variants of phosducin-like protein (PhLP) differ in the length of their N terminus and their expression pattern. The long form (PhLP<sub>L</sub>) is a ubiquitously expressed protein and binds G-protein βγ-subunits (Gβγ) and thereby inhibits Gβγ-mediated functions (1–5). The extended N terminus of PhLP<sub>L</sub> (83 amino acids) contains a highly conserved Gβγ-binding motif, which plays the crucial role in binding and regulating Gβγ-subunits (4, 6–11). In contrast, the short splice variant PhLP<sub>S</sub>, which has a more restricted expression, lacks this motif and did not seem to exert a major Gβγ inhibition, when tested with purified proteins. However, we recently demonstrated that PhLP<sub>S</sub> showed a more pronounced Gβγ inhibition than PhLP<sub>L</sub> in transiently transfected HEK 293 cells (12). Although PhLP<sub>L</sub> is the more abundantly expressed splice variant in most tissues, it is expressed at high levels in some tissues (e.g. adrenal gland) and has been suggested to play a role in regulation of catecholamine release (12, 13). This suggests that PhLP<sub>S</sub> has an effect on Gβγ that is not mediated by direct binding to Gβγ. Therefore, we set out to investigate this (potentially indirect) mechanism of Gβγ inhibition by PhLP<sub>S</sub> in intact cells. We report that transfection of PhLP<sub>S</sub> was associated with down-regulation of transfected and endogenously expressed Gβγ and that this down-regulation involved a proteasome-dependent pathway. It was recently reported that PhLP might inhibit the function of the cytosolic chaperonin complex (chaperonin complex containing TCP subunits (CCT)), which is involved in the folding of several proteins (14). Such an interaction might result in misfolding of G-protein subunits and might, thereby, provide an indirect mechanism of G-protein inhibition. In fact, we observed that PhLP<sub>S</sub> interacted with a subunit of the CCT complex and thereby appears to prevent proper folding of Gβγ complexes.

EXPERIMENTAL PROCEDURES

Materials—[32P]NAD and myo-[2-3H]inositol were purchased from PerkinElmer Life Sciences. Pertussis toxin was from Sigma. Primary antibodies used were rabbit polyclonal anti-PhLP-CT (12); rabbit polyclonal anti-PLCβ2, anti-Gβ, anti-Gβγ, and anti-Gγ1 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); rat monoclonal anti-TCP-1α from Calbiochem; and mouse monoclonal anti-β-actin from Sigma. Secondary antibodies were from Dianova (goat anti-rabbit and goat anti-mouse with horseradish peroxidase) or from Calbiochem (goat anti-rat horseradish peroxidase).

Construction of Expression Vectors—All of the cDNAs used in these studies were subcloned into pcDNA3 (Invitrogen). The cDNAs for PhLP<sub>L</sub>, PhLP<sub>S</sub>, and PhLP<sub>A18–20</sub> have been described previously (2, 12). The construction of deletion mutants and Trp-66 to Val mutants of PhLP<sub>S</sub> was done by PCR and confirmed by automated sequencing. In order to generate Gβ or Gγ subunits resistant to N-terminal destabilization, the dye-labeling protein O′-alkylguanine-DNA-alkyltransferase (AGT) (15), was subcloned N-terminal of either the Gβ or Gγ subunit in pcDNA3 by PCR and also confirmed by automated sequencing.

Phosphorylation of PhLP<sub>L</sub> and PhLP<sub>S</sub> (Gβγ-binding) Assays—PhLP<sub>L</sub>, PhLP<sub>S</sub>, and GST were purified from Escherichia coli as C-terminally His<sub>6</sub>-tagged proteins as described (4), and casein kinase 2 (CK2) kinase assays were performed with recombinant human CK2 enzyme (Boche Applied Science). A 100-μl reaction containing 5 μM of recombinant PhLP<sub>L</sub>, CK2 buffer (20 mM Tris-Cl, pH 7.5, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol), 0.3 milliunits of CK2, and 200 μM ATP (0.1 μCi of [32P]ATP for tracing) was incubated at 37 °C for 120 min. As controls,
HissD-tagged PhLP₅₁, PhLP₆₀, and GST were incubated under similar conditions but in the absence of CK2.

For binding assays, HEK cell extract was prepared by disrupting cells of one 10-cm dish in 1 ml of buffer (150 mM NaCl, 20 mM imidazole, 10 mM 2-mercaptoethanol, 2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, and Tris·Cl, pH 7.5), followed by centrifugation (20,000 × g, 10 min). Protein content of the supernatant was determined by the Bradford method. Binding was then performed by adding this supernatant from Gβγ-transfected HEK cells (100 μg of protein) to 400 nls of HissD-tagged PhLP₅₁, PhLP₆₀, or GST (phosphorylated or control-treated). For certain binding experiments, cell extract of PhLP isoform-transfected cells was combined with cell extract of Gβ₂·HissD·Gγ₇-expressing cells. After incubation (37 °C for 30 min), reactions were diluted with 5 volumes of ice-cold binding buffer supplemented with 50 μl of Ni²⁺-NTA-agarose (Qiagen) and rotated at 4 °C for 10 min. The beads were then washed, eluted with SDS-Laemmli buffer, and analyzed by SDS-PAGE and Western blotting (anti-Gβ antibody or anti-PhLP-CT antibody as indicated).

**Co-immunoprecipitation—**For the co-immunoprecipitation of PhLP constructs and endogenous TCP-1, HEK 293 cells were transiently transfected with the indicated cDNA and, 42 h later, cells were lysed in PBS, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (lysis buffer). Precipitation was performed with the PhLP-CT antibody precoated overnight with phosphoribosyltransferase and washed four times in lysis buffer, samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Detection of bound TCP-1 was performed with anti-TCP-1 antibody (rat; Calbiochem). For co-immunoprecipitation of AGT-Gβ₂ and AGT-Gγ₂, cells were transfected with AGT-Gβ₂ or AGT-Gγ₂ and were lysed and treated as before. Then AGT-Gβ₂ was precipitated with the Gβ₂ antibody prior to precipitation of Gγ₂ by PhLP-CT antibody. Elution from the resin was done with 100 mM sodium citrate, pH 3.0. After neutralization, samples were separated by SDS-PAGE and analyzed by Western blotting with the Gγ₂ antibody.

**ADP-ribosylation of G-protein α Subunits—**G-protein α, and βγ subunits were purified from bovine brain as described (16, 17). For the ADP-ribosylation, 4 mM Go, 0.6 mM Gβγ, and 100 ng of activated pertussis toxin were incubated in a 50-μl reaction according to the manufacturer's protocol. Elution from the resin was done with 100 mM sodium citrate, pH 3.0. After neutralization, samples were separated by SDS-PAGE and analyzed by Western blotting with the Gγ₂ antibody.

**Cell Culture and Transient Transfections—**Human embryonic kidney (HEK) 293 or HEK 293-TSA cells were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum and were kept in a 7% CO₂ humidified atmosphere. Usually, cells were transfected by using the CaPO₄ precipitation method or by electroporation. 10 cm dishes were washed twice with PBS and after washing from the DNA, and after changing to serum-free medium, cells were transfected at 40% confluence, and measurements were performed 6 h after transfection. In the case of membrane current measurements in HEK 293 cells stably expressing GIRQ1/4, transfecions were performed with the Effectene transfection kit (Qiagen) according to the manufacturer's protocol using 0.2 μg of CD8 cDNA as transfection reporter system (19). 0.2 μg of α₉/α₆-adrenergic receptor cDNA, and 0.6 μg of PhLP₅₁ cDNA or empty plasmid. Transfection efficiency was usually between 60 and 80% and was equal for the different PhLP isoforms as judged by transfection of different green fluorescent protein-tagged constructs. Control of protein expression by Western blotting was performed as described (12).

**Design and Use of RNA for TCP-1 Knock-down—**Two siRNAs directed against human TCP-1α were designed with the help of the MWG design tool (available on the World Wide Web at www.mwg-biotech.com) and the RNA sequence according to the Refseq human data base at NCBI (accession number NM_030752). The targeted sequences were 5′-GAA GUU GGU GGA GAU GGA ACU ATT (positions 272–292) and 5′-GAA GUU GUA CAG GAG AGA ATT (positions 1067–1087). They were run on a Blast search and found to interact only with human TCP-1α. They were produced (MWG) as preannealed duplex RNAs (sense and antisense each ending with ddiT). For control experiments, siRNA directed against GFP was designed as described recently (5′-CGU AAA CGG CCA GUU CTT, positions 64–84 (20)). Transfection of HEK 293-TSA cells with the siRNAs was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and analyzed 42 h later. In pilot experiments, both specific anti-TCP-1α siRNAs (in concentrations between 40 and 80 pmol/transfection in a 12-well plate) were found to be effective in reducing the amount of TCP-1α by roughly 50–70% as determined by Western blots (data not shown). In all further experiments, they were subsequently used in combination (at 40 pmol/well each; for simplification named siTCP-1α). siRNA against GFG was used in parallel at a concentration of 80 pmol/well.

**Measurement of GIRK Channel Currents and Inositol Phosphate Generation—**Membrane currents were recorded under voltage clamp conditions, using conventional whole cell patch clamp techniques exactly as described recently (21). Iₐ was measured as an inward current using a holding potential of –90 mV, and voltage ramps (from –120 to +60 mV in 500 ms, every 10 s) were used to determine current-voltage (I-V) relationships. All measurements were performed at room temperature, and summarized results are presented as mean ± S.E. Student's t test was done to test for significance of differences. Inositol phosphate measurements were performed in triplicates and results were analyzed as means ± S.E. of at least three independent experiments. Analysis of variance and post-test comparison (Bonferroni) were done as appropriate.

**RNA Preparation, Reverse Transcription, and Quantitative PCR—**For total RNA preparation, cells seeded on 6-well plates were lysed and treated according to the instructions of the RNeasy Mini Kit (Qiagen) and RNA was precipitated with 2 M EDTA·Na₂, and alcohol. The concentration was determined on a denaturing formaldehyde gel. One μg of total RNA of each sample was reverse-transcribed with the Superscript III Reverse Transcriptase-Kit (Invitrogen) using oligo(dT) to produce first strand cDNA. For PCR of the human Gβ₂ cDNA, the primers were chosen to distinguish the cDNA product (95 bp) from a potentially contaminating genomic DNA product (324 bp): (a) 5′-CTTGGGATGGAGCTGACGCGGTTGTCAT-3′ and (b) 5′-ATGGAGCGGCTGATCAAAG (c) 5′-CTTGGGATGGAGCTGACGCGGTTGTCAT-3′ and (b) 5′-CAAATTCTGGTCATCCATAC-3′, resulting in a 144-bp product. Real-time PCR (in triplicates) was performed on an ABI PRISM Sequence Detection System 7700 (Applied Biosystems) with Sybr Green (Cambrex) as fluorescent, 6-carboxy-X-rhodamine (Molecular Probes, Inc., Eugene, OR) as reference dye and Hot-Taq (Eppendorf) polymerase. PCR conditions were 95°C for 10 s, 60°C for 1 min, and 72°C for 1 min. A standard curve was generated with several dilution factors of the cDNA template. A triplicate analysis of each sample and primer set was performed. The Cq (crossing point) was defined as the point of fluorescence becoming significant compared with baseline fluorescence. The average Cq was used to calculate the relative amount of cDNA and the Ct (cycle threshold) was used as a quantitative measure of cDNA levels. The relative expression of cDNA was calculated using the 2^-ΔCt formula. The data is expressed as the fold change relative to the control.

**RESULTS**

**Effects of Phosphorylation of PhLP by CK2 on Gβγ Binding—**We have recently demonstrated that PhLP₁ is constitutively phosphorylated by CK2 and that this phosphorylation inhibited the effect of PhLP on Gβγ-mediated inositol phosphate generation in intact HEK 293 cells (12). The homologous protein phosphducin is also phosphorylated at the N terminus by several different kinases, and these phosphorylations lead to a loss in affinity toward Gβγ subunits (11). We therefore tested whether CK2-mediated phosphorylation affected directly Gβγ-binding of PhLP₁. Recombinant C-terminally His₄-tagged PhLP₁, was purified from E. coli by Ni²⁺-NTA affinity purification and was phosphorylated with recombinant CK2. Completion of phosphorylation was monitored and controlled by a mobility shift of the Coomassie-stained PhLP₁ band in SDS-PAGE (Fig. 1A, upper panel). Equimolar concentrations (400 nm) of PhLP₁, phosphorylated PhLP₁, PhLP₁-His₄, or GST-His₄, were incubated with lysates from HEK 293 cells overexpressing Gβγ subunits. The His₄-tagged proteins were then precipitated with Ni²⁺-NTA-agarose and the amounts of coprecipitated Gβγ subunits were monitored by Western blotting with a Gβγ-specific antibody (Fig. 1A, lower panel). These experiments showed that Gβγ subunits were coprecipitated in a complex with PhLP₁ as well as with phosphorylated PhLP₁, but there was no change in the amount of coprecipitated Gβγ after phosphorylation of PhLP₁. In contrast, PhLP₁ bound to Gβγ only weakly. These findings were confirmed in the effects of PhLP on pertussis toxin-catalyzed ADP-ribosylation of G-protein α, subunits (Go₄,). This ADP-ribosylation is stimulated
by the presence of $\beta\gamma$ subunits. As depicted in Fig. 1B, the $G\beta\gamma$-stimulated ADP-ribosylation of $G_{o\gamma}$ was inhibited by PhLP$_1$ and by phospho-PhLP$_1$ to a similar extent, whereas the inhibition by PhLP$_2$ was only partial. IC$_{50}$ values for PhLP$_1$ and PhLP$_2$ ($18.7 \pm 4.5$ and $160 \pm 44.6$ nM, respectively) were within the previously published range (4) but were not different between PhLP$_1$ and phospho-PhLP$_1$ ($16.5 \pm 5.2$ nM). Therefore, a decrease in the $G\beta\gamma$ binding affinity of CK2-phosphorylated wild-type PhLP$_1$ did not seem to account for its reduced capacity to inhibit $G\beta\gamma$-stimulated inositol phosphate generation in intact cells.

$G\beta\gamma$-binding by PhLP$_1$ Is Dependent on Trp-66 in Its N Terminus—To further analyze the $G\beta\gamma$ regulatory role of PhLP$_1$ in intact cells, we mapped the $G\beta\gamma$-interacting sites. In the homologous retinal protein phosducin, the exchange of a tryptophan to a valine (Trp-29 to Val) within a conserved helical region of phosducin and PhLPL (8) (Fig. 1A). This region forms the $\alpha$-helix 1 of the N terminus of these proteins (8). The essential tryptophan (W) marked in boldface type is at position 66 in rat PhLP$_1$ and at position 29 in rat phosducin (upper panel). In the lower panel, a representative Western blot of binding experiments is shown, where the different PhLP$_1$ mutants expressed in HEK 293 cells were transiently transfected with cDNAs of PhLPL, PhLPLW66V, and PhLP$_1$ on the pertussis toxin-dependent (100 ng) and $G\beta\gamma$-dependent (6 nM) $[^{32}P]ADP$-riboside transfer onto purified $G_{o\gamma}$ (4 nM) was analyzed as described under “Experimental Procedures.” As control, CK2 was added. Shown are the results of 3–7 independent experiments. Maximal inhibition ± S.E. was as follows: for PhLP$_1$, $90.2 \pm 1.8\%$ (n = 7); for phospho-PhLP$_1$, $98.6 \pm 0.3\%$ (n = 6); for PhLP$_2$, $22.6 \pm 5.2\%$ (n = 4). The IC$_{50}$ values for PhLP$_1$ and phospho-PhLP$_1$ were $18.7 \pm 4.5$ and $16.5 \pm 5.2$ nM, respectively, and $160 \pm 44.6$ nM for PhLP$_2$. C. Alignment of the conserved $G\beta\gamma$-binding motif from rat PhLP$_1$ (rat PhLP), rat phosducin (rat Phd), and PhLP from Drosophila melanogaster (dro PhLP). This region forms the $\alpha$-helix 1 of the N terminus of these proteins (8). The essential tryptophan (W) marked in boldface type is at position 66 in rat PhLP$_1$ (Fig. 1A), the effect of increasing amounts of PhLP$_1$, phospho-PhLP$_1$, and PhLP$_2$ on the pertussis toxin-dependent (100 ng) and $G\beta\gamma$-dependent (6 nM) $[^{32}P]ADP$-riboside transfer onto purified $G_{o\gamma}$ (4 nM) was analyzed as described under “Experimental Procedures.” As control, CK2 was added. Shown are the results of 3–7 independent experiments. Maximal inhibition ± S.E. was as follows: for PhLP$_1$, $90.2 \pm 1.8\%$ (n = 7); for phospho-PhLP$_1$, $98.6 \pm 0.3\%$ (n = 6); for PhLP$_2$, $22.6 \pm 5.2\%$ (n = 4). The IC$_{50}$ values for PhLP$_1$ and phospho-PhLP$_1$ were $18.7 \pm 4.5$ and $16.5 \pm 5.2$ nM, respectively, and $160 \pm 44.6$ nM for PhLP$_2$. C. Alignment of the conserved $G\beta\gamma$-binding motif from rat PhLP$_1$ (rat PhLP), rat phosducin (rat Phd), and PhLP from Drosophila melanogaster (dro PhLP). This region forms the $\alpha$-helix 1 of the N terminus of these proteins (8). The essential tryptophan (W) marked in boldface type is at position 66 in rat PhLP$_1$ and at position 29 in rat phosducin (upper panel). In the lower panel, a representative Western blot of binding experiments is shown, where the different PhLP$_1$ mutants expressed in HEK 293 cells were transiently transfected with cDNAs of PhLPL, PhLP$_1$, PhLP$_2$, and glutathione S-transferase (GST; 9.59 µg/ml) were incubated with lysate from HEK 293 cells transfected with G$\beta\gamma$, and G$\gamma$, cDNA. After incubation for 30 min at 37 °C, the recombinant proteins were precipitated with Ni$^{2+}$-NTA-agarose, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The phosphorylation state of PhLP$_1$ was controlled by the mobility shift of the Coomassie-stained band (12). Western blot detection was performed with a G$\beta\gamma$-specific antibody and showed equal expression of the indicated proteins.

FIG. 1. Binding of PhLP isoforms to G$\beta\gamma$. A, binding of PhLP to G$\beta\gamma$. Equimolar concentrations (400 nM) of recombinant C-terminally His$_6$-tagged PhLP$_1$ (14.04 µg/ml), phosphorylated PhLP$_1$, (14.04 µg/ml), PhLP$_2$ (10.3 µg/ml), and glutathione S-transferase (GST; 9.59 µg/ml) were incubated with lysate from HEK 293 cells transfected with G$\beta\gamma$, and G$\gamma$, cDNA. After incubation for 30 min at 37 °C, the recombinant proteins were precipitated with Ni$^{2+}$-NTA-agarose, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The phosphorylation state of PhLP$_1$ was controlled by the mobility shift of the Coomassie-stained band (12). Western blot detection was performed with a G$\beta\gamma$-specific antibody (IB: G$\beta\gamma$). Shown is a blot representative of three independent experiments and the corresponding Coomassie stain of the gel.
of PhLPL to G

phosphate formation was determined. Data represent mean ± S.E. of 3–6 independent experiments (analysis of variance: p < 0.0001; Bonferroni: p < 0.001 for all versus control). Shown is also a Western blot (lower panel) comparing the expression of the indicated constructs. To control expression, N-terminal constructs were tagged with a FLAG epitope. Tris/Tricine gels were loaded with 20% of the cell lysate from one well of a 6-well plate from the transfection, and detection was performed with the M2 FLAG antibody (IB: Flag).

FIG. 2. The first 83 amino acids of PhLP are sufficient to inhibit Gβγ-mediated inositol phosphate generation. A, alignment of different N-terminal constructs of PhLP. The constructs are named by the position of amino acids in PhLPL and are aligned to the topology model derived from the crystal structure of the phosducin-Gβγ complex (Ref. 8; α-helices 1 (H1), 2 (H2), and 3 (H3) with their related Gβγ binding sites (black lines)). B, HEK 293 cells were transiently transfected with the indicated constructs (8 μg/10-cm dish), and inositol phosphate formation was determined. Data represent mean ± S.E. of 3–6 independent experiments (analysis of variance: p < 0.0001; Bonferroni: p < 0.001 for all versus control). In addition, Gβγ′-mediated inositol phosphate generation to the same extent as the wild-type PhLP, PhLP A18–20, and PhLP AV compared with wild-type PhLP (**, p < 0.001 versus control; ##, p < 0.01 versus PhLP). B, Western blot representative of 5–7 independent experiments demonstrating significant down-regulation of Gβγ inhibition by PhLP, PhLP A18–20, and PhLP AV in comparison with control or wild-type PhLP. The protein levels of PLCβ2 (IB: PLCβ2) remained constant. C, effect of lactacystine (4 μM for 4 h at 37 °C) on the protein level of Gγ2 (IB: Gγ2) in control (ctrl) and PhLP AV-transfected HEK 293 cells. The analysis was performed in three independent experiments (**, p < 0.001 versus control untreated (–); #, p < 0.05 versus PhLP untreated (–)). Also shown is a representative Western blot.

FIG. 3. PhLP, PhLP A18–20, or PhLP AV down-regulate Gβ and Gγ. A, inhibition of inositol phosphate generation. HEK 293 cells were transiently transfected as in Fig. 1D, but the specific amount of cDNA was lowered to 0.5 μg/10-cm dish. The determination of inositol phosphates with the indicated cDNAs demonstrates the high potency of Gβγ inhibition by PhLP, PhLP A18–20, and PhLP AV compared with wild-type PhLP (**, p < 0.001 versus control; ##, p < 0.01 versus control; ##, p < 0.01 versus PhLP). B, Western blot representative of 5–7 independent experiments demonstrating significant down-regulation of Gβ (IB: Gβ) and Gγ (IB: Gγ′) in the presence of PhLP, or the phosphorylation-deficient mutants PhLP A18–20 and PhLP AV in comparison with control or wild-type PhLP. The protein levels of PLCβ2 (IB: PLCβ2) remained constant. C, effect of lactacystine (4 μM for 4 h at 37 °C) on the protein level of Gγ2 (IB: Gγ2) in control (ctrl) and PhLP AV-transfected HEK 293 cells. The analysis was performed in three independent experiments (**, p < 0.001 versus control untreated (–); #, p < 0.05 versus PhLP untreated (–)). Also shown is a representative Western blot.
of PhLP (Fig. 2A) and tested their ability to inhibit Gβγ-mediated inositol phosphate generation (Fig. 2B). Expression of the diverse constructs was comparable (Fig. 2B, lower panel). We found that the N-terminal constructs (containing at least the first 83 amino acids of PhLPL) inhibited Gβγ-stimulated signaling to a similar extent as did the wild-type full-length PhLPL in intact cells (by about 40–50%).

PhLPs and Phosphorylation-deficient PhLPL Down-regulate Gβγ Protein—Looking at the effect of PhLPs and of both phosphorylation-deficient mutants PhLP1A18–20 and PhLP3AV (Fig. 3A), we found that not only PhLPs but also the two mutants were able to inhibit the inositol phosphate signal more effectively than wild-type PhLPL. This was seen although direct binding of Gβγ to PhLPs and PhLP3AV (which bears the Trp-66 to Val
mutation in addition to the A18–20 mutation) was less than that of wild-type PhLP$_2$ (Fig. 1C). We therefore concluded that a different type of regulation on G$\gamma$/H9252/H9253 subunits must exist in cells, which is distinct from direct G$\gamma$/H9252/H9253 binding. In line with this conclusion, the protein content of both G-protein subunits, G$\beta$ and G$\gamma$, were dramatically decreased in HEK 293 cells co-transfected with PhLP$_2$A18–20, PhLP$_2$AV, or PhLP$_2$ (Fig. 3B). As a control, the protein level of PLC$\beta_2$ was unchanged (Fig. 3B, lower panel). The decrease in the amount of G$\gamma_2$ by PhLP$_2$ was partially restored by a 4-h incubation with the specific proteasome inhibitor lactacystine, suggesting the involvement of proteasomal degradation in the PhLP$_2$-induced G$\gamma$ decrease (Fig. 3C).
The Fate of \( \gamma \) Subunits—To analyze whether the PhLP\(_s\)-mediated decrease in \( \gamma \) subunit levels accounted for the apparent inhibition of \( \gamma \)-mediated inositol phosphate signaling, we attempted to prevent \( \gamma \) degradation by stabilization of \( \gamma \) subunit. Recently, it was demonstrated that the \( \gamma \) subunit is a substrate for ubiquitylation and degradation via the N-end rule pathway in bovine brain (27). That means that posttranslational modification of the N terminus leads to susceptibility to proteasome-dependent degradation. It was also demonstrated that mutation of the N terminus could protect the \( \gamma \) subunit from degradation. We therefore stabilized the \( \gamma \) subunit by N-terminal fusion to the dye-labeling protein 6-alkylguanine-DNA-alkyltransferase, AGT (15). These modifications did not prevent functional interaction with PLC\( \beta \_2 \) (see below). In line with previous observations, this modification stabilized \( \beta \) as well as \( \gamma \) subunits (27) and prevented \( \beta \) and \( \gamma \) from disappearance in the presence of PhLP\(_s\) (Fig. 4A). However, expression of the AGT-stabilized N-terminal constructs of \( \beta \) and \( \gamma \) either with the wild-type partner or in combination did not change the pattern of functional inhibition seen with PhLP\(_s\) (Fig. 4B). These experiments also illustrate that the AGT-modified \( \beta \) and \( \gamma \) subunits were functional, since their transfection increased the inositol phosphate generation, although less than the wild-type forms (compare control (ctr) to basal in each panel). Taken together, PhLP\(_s\) led to down-regulation of \( \beta \gamma \) dysfunction in the presence of PhLP\(_s\).
ence of PhLP$_S$ we analyzed the interaction of G$\beta$ and G$\gamma$ by co-precipitation. In contrast to PhLP$_L$, expression of PhLP$_S$ led to a strongly decreased interaction of G$\beta$ with G$\gamma$ as determined by the failure of precipitated AGT-G$\beta_2$ to co-precipitate AGT-G$\gamma_2$ in the presence of PhLP$_S$ (Fig. 4C). Since efficient interaction of G$\beta$ and G$\gamma$ is indicative of the proper folding of the G$\beta$ subunit (28), the lack of G$\beta$-G$\gamma$ interaction is suggestive of G$\beta$ misfolding induced by PhLP$_S$.

Structural Determinants in PhLP$_S$ for G$\beta\gamma$ Down-regulation—We further analyzed the mechanism of PhLP$_S$ on G$\beta\gamma$ and therefore consecutively truncated the N terminus of PhLP$_S$ to investigate the structural requirements for the G$\beta\gamma$-down-regulating effect of PhLP$_S$ in intact cells (Fig. 5A). The C-terminal constructs PhLP 119–301 and PhLP 132–301 were able to inhibit inositol phosphate generation to the same extent as was PhLP$_S$ (Fig. 5B). Further truncating the N terminus (PhLP 145–301) decreased G$\beta\gamma$-regulating effects. We therefore concluded that the ability of PhLP$_S$ to regulate G$\beta\gamma$ was dependent on the function of $\alpha$-helix 3 together with the C-terminal half of PhLP. Recently, it was reported that PhLP$_L$ can bind and inhibit the cytosolic chaperonin complex (CCT; a hexadecamer consisting of eight different subunits) via binding to one of its subunits named TCP-1 (14). It was demonstrated that PhLP$_S$ inhibited the folding of luciferase and the G$_\alpha$ protein $\alpha$ subunits, but a role in G$\beta$ folding has not been addressed so far. To analyze whether TCP-1-a also interact with PhLP$_S$, we performed co-immunoprecipitations of TCP-1-a with PhLP$_S$. Immunoprecipitation of PhLP$_S$ and the C-terminal constructs revealed that TCP-1-a interacted with the same constructs that caused inositol phosphate inhibition (Fig. 5C) and G$\beta\gamma$ down-regulation (data not shown). In order to investigate whether TCP-1-a plays a role in the folding of G$\beta\gamma$, we performed additional knock-down experiments with the help of siRNA technology (for a review see Ref. 29). Fig. 5D shows a representative experiment where the specific knock-down of endogenous TCP-1-a in HEK-TSA cells led to down-regulation of transfected G$\gamma_2$ subunits. Cells transfected with siRNA (siTCP-1-a or, for control, siGFP) were unaffected in terms of expression of other transfected (PLC$\beta_2$) or endogenous proteins ($\beta$-actin). This finding is in agreement with the function of TCP-1-a in regulating G$\beta$-folding (Fig. 7). We further show that inhibition of, most likely, G$\beta$-degradation and inhibits its effects on a G$\beta\gamma$-dependent effector such as PLC$_B_2$ (1). A second type of G$\beta$ regulation renders newly synthesized G$\beta$ subunits nonfunctional, leading to the degradation of G$\beta$ subunits. This effect appears to be mediated by an interaction with the TCP-1-a subunit of CCT (2), which is involved in the folding of WD40 repeat proteins (like the G$\beta$ subunit). Switching between the two mechanisms is exerted either by alternative splicing (since PhLP$_S$ only weakly binds to G$\gamma$) or by CK2-dependent phosphorylation of PhLP (since P-PhLP$_S$ does not inhibit CCT).

FIG. 7. Proposed mechanism of G$\beta\gamma$ subunit regulation by PhLP: Two faces of a regulator. The constitutively phosphorylated long isoform PhLP$_L$ binds to G$\beta\gamma$ and inhibits its effects on a G$\beta\gamma$-dependent effector such as PLC$_B_2$ (1). A second type of G$\beta$ regulation renders newly synthesized G$\beta$ subunits nonfunctional, leading to the degradation of G$\beta$ subunits. This effect appears to be mediated by an interaction with the TCP-1-a subunit of CCT (2), which is involved in the folding of WD40 repeat proteins (like the G$\beta$ subunit). Switching between the two mechanisms is exerted either by alternative splicing (since PhLP$_S$ only weakly binds to G$\gamma$) or by CK2-dependent phosphorylation of PhLP (since P-PhLP$_S$ does not inhibit CCT).

DISCUSSION

In this work, we show that inhibition of G$\beta\gamma$-mediated signaling by PhLP occurs through at least two different mechanisms: (a) through direct binding by a high affinity N-terminal G$\beta\gamma$ binding site (PhLP$_L$; Figs. 1, 2, and 7) or (b) through inhibition of, most likely, G$\beta\gamma$ folding and subsequent down-regulation of G$\beta$ and G$\gamma$ protein levels (Figs. 3, 4, and 7). The down-regulation (as a putative indicator of misfolded G$\beta\gamma$) was a specific phenomenon seen with PhLP$_L$ and the phosphorylation-deficient mutants of PhLP$_L$ (bearing the A18–20 mutation). This finding would imply that regulation of PhLP (either by dephosphorylation of the constitutively phosphorylated PhLP$_L$ or by alternative splicing to produce PhLP$_S$) can switch the G$\beta\gamma$-regulatory function of PhLP from direct binding toward inhibition of G$\beta\gamma$-folding (Fig. 7). We further show that the diminished protein levels of G$\beta$ and G$\gamma$ were sensitive to modification of the N terminus. This finding is in agreement with a recent report that the G$\gamma$ subunit is subject to regulation by the proteasome via the N-end rule pathway (27). In line with this, we observed a partial reversal of the down-regulation by PhLP$_S$ in intact cells by the proteasome-specific inhibitor lactacystine (Fig. 3C). We also show that endogenous G$\beta$ was effectively down-regulated by PhLP$_L$ and that this resulted in inhibition of PLC$\beta$ and receptor-activated GIRK activity.

Recently, it was reported that PhLP$_L$ selectively inhibited the function of CCT, which is involved in the folding of actin, tubulin, and G$\alpha$ subunits but has been suggested to participate, more importantly, in folding of WD40 repeat proteins like the G-protein $\beta$ subunit (33). However, a role in G$\beta\gamma$ subunit folding remained to be elucidated (14). We now demonstrate
that the siRNA-induced gene silencing of the CCT subunit TCP-1α resulted in a reduction of both endogenous Gβ2 and co-transfected Gβγ subunits. Since Gβγ down-regulation was the prominent feature seen with PhLPγ, this observation suggests that PhLPγ-dependent Gβγ-down-regulation occurred via inhibition of TCP-1α-mediated Gβγ folding. To add further evidence to this contention, we show that the Gβγ-down-regulating domains of PhLPγ and the TCP-1α-binding domains are identical (Fig. 5).

Given the fact that PhLPγ was constitutively phosphorylated by CK2 in HEK cells and diverse tissue types (12), and combined with the observation that phosphorylated PhLPγ was not able to down-regulate Gβγ, inhibiting the function of CCT could be the crucial step in regulating Gβγ function, when PhLP is dephosphorylated or alternatively spliced. Thus, PhLP can inhibit Gβγ-mediated effects via two entirely different mechanisms: direct Gβγ binding and impairment of Gβγ folding. The switch between these two mechanisms is regulated either at the level of PhLP synthesis by alternative splicing or at the post-translational level by CK2-dependent phosphorylation (Fig. 7). Such a switch between two entirely different types of regulation is, to our knowledge, without precedent and illustrates that PhLP is a unique and complex regulator of G-protein-mediated signaling.

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Phosducin-like Protein Regulates G-Protein γ Folding by Interaction with Tailless Complex Polypeptide-1 α: DEPHOSPHORYLATION OR SPLICING OF PhLP TURNS THE SWITCH TOWARD REGULATION OF G βγ FOLDING

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