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Phosducin-like protein acts as a molecular chaperone for G protein βγ dimer assembly

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Phosducin-like protein (PhLP) is a widely expressed binding partner of the G protein βγ subunit dimer (Gβγ). However, its physiological role is poorly understood. To investigate PhLP function, its cellular expression was blocked using RNA interference, resulting in inhibition of Gβγ expression and G protein signaling. This inhibition was caused by an inability of nascent Gβγ to form dimers. Phosphorylation of PhLP at serines 18–20 by protein kinase CK2 was required for Gβγ formation, while a high-affinity interaction of PhLP with the cytosolic chaperonin complex appeared unnecessary. PhLP bound nascent Gβ in the absence of Gγ, and S18–20 phosphorylation was required for Gγ to associate with the PhLP-Gβ complex. Once Gγ bound, PhLP was released. These results suggest a mechanism for Gβγ assembly in which PhLP stabilizes the nascent Gβ polypeptide until Gγ can associate, resulting in membrane binding of Gβγ and release of PhLP to catalyze another round of assembly.

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Introduction

Heterotrimeric G proteins mediate a wide array of hormonal, neuronal and sensory signals that control numerous physiological processes ranging from cardiac rhythm (Rockman *et al*, 2002) to psychological behavior (Gainetdinov *et al*, 2004) to vision (Arshavsky *et al*, 2002). G protein signaling is initiated by the binding of a ligand to the extracellular face of a G protein-coupled receptor (GPCR), resulting in a change in the packing of the seven-transmembrane α-helices found in all GPCRs. This conformational change activates the G protein on the intracellular surface of the receptor by initiating an exchange of GDP for GTP on the G protein α subunit (Gα). GTP binding causes Gα to dissociate from the G protein βγ subunit complex (Gβγ). Both Gα·GTP and Gβγ control the activity of effector enzymes and ion channels that determine the intracellular concentration of second messengers (cyclic nucleotides, inositol phosphates, Ca^{2+} and K^{+}), which in turn orchestrate the cellular response to the stimulus.

Phosducin-like protein (PhLP) is a widely expressed member of the phosducin gene family that is believed to participate in G protein signaling by virtue of its ability to bind the Gβγ dimer with high affinity (Miles *et al*, 1993; Thibault *et al*, 1997; Savage *et al*, 2000; Schroder and Lohse, 2000). Phosducins were originally thought to downregulate G protein pathways by sequestering Gβγ from its interaction with Gα (Bauer *et al*, 1992; Lee *et al*, 1992; Yoshida *et al*, 1994). However, the results of recent studies have not been consistent with this putative role. Specifically, disruption of the *PhLP1* gene in the chestnut blight fungus *Cryphonectria parasitica* (Kasahara *et al*, 2000) and in the soil amoeba *Dictyostelium discoideum* (Blaauw *et al*, 2003) yielded the same phenotype as the disruption of the Gβ gene. Moreover, PhLP deletion blocked G protein signaling in *Dictyostelium* (Blaauw *et al*, 2003). In another study, the duration of opiate desensitization was prolonged in mice in which PhLP expression in the brain was inhibited by antisense oligonucleotide treatment (Garzon *et al*, 2002). All of these observations are the exact opposite of what would be expected if PhLP were a negative regulator. As a result, they have led to the conclusion that PhLP must be a positive regulator of G protein signaling.

Insight into possible ways in which PhLP might facilitate G protein function has come from the observation that PhLP interacts with the cytosolic chaperonin complex (CCT), an essential molecular chaperone that mediates the folding of actin, tubulin and other proteins into their native structures (McLaughlin *et al*, 2002b). PhLP was shown to interact with CCT as a regulator and not as a folding substrate. In addition, the cryoelectron microscopic structure of the PhLP-CCT complex (Martín-Benito *et al*, 2004) shows that PhLP binds CCT at the top of the CCT apical domains positioned above the folding cavity in a manner analogous to prefoldin, a CCT cochaperone that binds nascent actin polypeptide chains and delivers them to CCT for folding (Martin-Benito *et al*, 2002). Coupling these findings with the fact that yeast Gβ (Ho *et al*, 2002) and other proteins with seven β-propeller structures similar to Gβ (Valpuesta *et al*, 2002; Camasses *et al*, 2003) interact with CCT suggests that PhLP might function as a chaperone for the folding of Gβ. To test this notion, the effects of small interfering RNA (siRNA)-mediated inhibition of PhLP expression in human cell lines on G protein signaling, Gβγ expression and assembly of nascent Gβγ dimers were determined, as were the effects of overexpression of PhLP and several PhLP variants lacking either protein kinase CK2 (CK2) phosphorylation sites, Gβγ binding or CCT binding. The results show that PhLP is required for the formation of the

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Gβγ complex, and they outline a mechanism by which PhLP catalyzes Gβγ dimer assembly.

Results

Cellular depletion of PhLP inhibits Gβ expression and G protein signaling

To gain insight into the physiological function of PhLP, siRNA was used to block its expression in HeLa cells. Two different siRNA sequences were prepared and are referred to as PhLP-A and PhLP-B siRNA. Cells were transfected with either of these siRNAs, or a control siRNA targeting lamin A/C, and PhLP protein expression was determined by immunoblotting. PhLP-A siRNA was modestly effective, inhibiting PhLP protein expression by 50% (Figure 1A), while PhLP-B siRNA was much more effective, blocking PhLP expression by 90%. The control lamin A/C siRNA had no effect on PhLP expression when compared to mock-transfected cells, yet it inhibited lamin A/C expression by 90%. Likewise, the PhLP siRNAs had no effect on lamin A/C expression, indicating that the siRNAs were acting specifically to reduce their target mRNAs.

Based on the lack of G protein signaling upon PhLP deletion in single-celled organisms (Kasahara *et al*, 2000; Blaauw *et al*, 2003), it was reasonable to suspect that siRNA-mediated depletion of PhLP would adversely affect G protein signaling in HeLa cells. As a first step to investigate this idea, the expression of the $G\beta_1$ subunit, the most widely and abundantly expressed Gβ subunit, was determined in PhLP-depleted cells. PhLP-A siRNA had no detectible effect on $G\beta_1$ expression in HeLa cells. However, the more potent PhLP-B siRNA consistently decreased $G\beta_1$ expression by 40%, demonstrating that removal of 90% of the PhLP from the cell somehow inhibited endogenous $G\beta_1$ expression.

Such a decrease in $G\beta_1$ levels would be expected to impact G protein signaling. This notion was investigated by measuring the change in intracellular Ca^{2+} in HeLa cells in response to histamine. Histamine receptors initiate a classical G_q-mediated cascade, which results in an influx of Ca^{2+} into the cytosol (Bootman *et al*, 1997). PhLP-B siRNA transfection caused a 60% reduction in histamine-induced Ca^{2+} transient compared to lamin A/C siRNA or mock-transfected cells (Figure 1B). PhLP-A siRNA treatment also caused a modest but reproducible decrease in Ca^{2+} influx. Thus, it appears that PhLP acts as a positive regulator of G protein signaling in HeLa cells by contributing to the cellular expression of $G\beta_1$.

The PhLP siRNA-mediated impairment of $G\beta_1$ expression could potentially result from a loss of PhLP function at any level of the expression process from gene transcription to protein degradation. To assess pretranslational events, the effect of PhLP depletion on $G\beta_1$ mRNA levels was determined by Northern blotting. Treatment of HeLa cells with either of the PhLP siRNAs caused no change in the levels of $G\beta_1$ mRNA compared to lamin A/C or mock-transfected controls

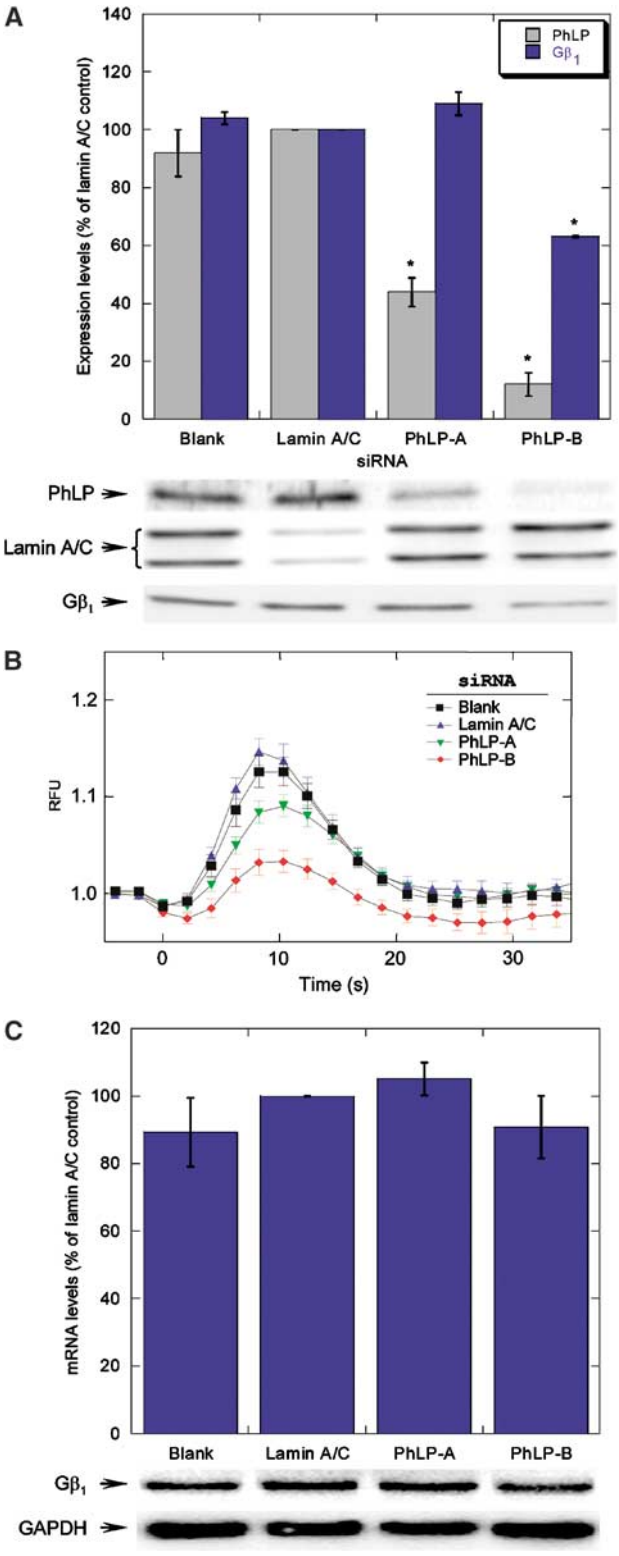

Figure 1 siRNA-mediated depletion of PhLP inhibits Gβ expression and G protein signaling. (**A**) HeLa S3 cells were treated with the indicated siRNAs and then assayed after 96 h. Protein expression of PhLP, $G\beta_1$ and lamin A/C were determined by immunoblotting. PhLP and $G\beta_1$ band intensities were quantified and expressed as a percentage of the lamin A/C sample. Bars and symbols in each panel represent the average ± s.e. from three separate experiments. Statistical significance relative to the lamin A/C control was determined by a paired *t*-test (*$P<0.01$). (**B**) Changes in intracellular Ca^{2+} in living cells were determined by measuring fluorescence from a Ca^{2+}-sensitive dye using a FlexStation plate reader. Histamine (50 nM) was added at time zero and measurements were taken at the times indicated. Fluorescence data were normalized to the signal prior to histamine addition. (**C**) Levels of $G\beta_1$ and GAPDH mRNA were determined by Northern blotting. Band intensities were quantified, normalized to that of GAPDH and expressed as a percentage of the lamin A/C sample.

(Figure 1C), indicating that PhLP acts translationally or post-translationally to promote $G\beta_1$ expression.

PhLP is required for Gβγ dimer assembly

Two recent studies have suggested that PhLP might be involved in post-translational regulation of Gβ folding or Gβγ assembly (Blaauw *et al*, 2003; Martín-Benito *et al*, 2004). This idea was based on several observations including the similarity of the PhLP-CCT structure to another CCT cochaperone, prefoldin (Martín-Benito *et al*, 2004), and on the mislocalization of Gβ-GFP and Gγ-GFP to the cytosol when the *PhLP1* gene was deleted in *Dictyostelium* (Blaauw *et al*, 2003). These observations lead to an examination of the effect of siRNA-mediated PhLP depletion on the expression of the Gβγ dimer. HEK-293 cells were chosen for this experiment because they readily overexpress Gβ and Gγ from plasmid vectors. Cells were siRNA treated and then cotransfected 24 h later with $G\beta_1$ and N-terminally hemagglutinin (HA)-tagged $G\gamma_2$. The HA-$G\gamma_2$ was immunoprecipitated 72 h later and the co-immunoprecipitate was immunoblotted with anti-$G\beta_1$ and anti-HA antibodies to determine the amount of Gβγ complex formed. PhLP-A and -B siRNA treatment decreased PhLP expression in these cells by 25 and 75%, respectively, compared to lamin A/C controls (Figure 2A). These siRNAs also decreased the amount of Gβγ complex by 35 and 75%, respectively (Figure 2A). The close correlation between the inhibition of PhLP expression and the decrease in Gβγ levels in a second human cell line further demonstrates the need for PhLP in Gβγ expression.

The data in Figure 1 suggest that the decreases in Gβ and Gγ expression were caused by effects of PhLP depletion on translation or post-translation events. To determine whether this was also the case in the overexpression system, the effect of PhLP depletion on overexpressed Gβ and Gγ mRNA levels were measured. No significant differences were observed (data not shown), confirming that PhLP depletion had little effect on pretranslational events.

Given the observations suggesting a role for PhLP in Gβγ assembly (Blaauw *et al*, 2003; Martín-Benito *et al*, 2004), it seemed reasonable to explore the effects of PhLP depletion on this process. The rate of assembly of nascent Gβγ dimers was measured in a pulse-chase experimental format designed to detect newly synthesized proteins. HEK-293 cells that had been treated with PhLP-B siRNA and then transfected with Flag-$G\beta_1$ and HA-$G\gamma_2$ were pulsed with [^{35}S]methionine for 10 min, and then chased for the times indicated with excess unlabeled methionine. At the end of the chase period, the amount of Gβγ dimer formed was determined by immunoprecipitating the HA-Gγ and measuring the amount of co-immunoprecipitating [^{35}S]-labeled Gβ. In lamin A/C siRNA-treated cells, there was a clear increase in [^{35}S]Gβ in the Gγ immunoprecipitate as the chase time increased (Figure 2B). In contrast, there was almost no increase in co-immunoprecipitation of [^{35}S]Gβ during the chase period in cells treated with the PhLP-B siRNA. In addition, the amount of Gγ synthesized during the pulse was reduced two-fold in the PhLP-B-treated cells. The observed decrease in [^{35}S]Gβ co-immunoprecipitation in the PhLP-B-treated cells was clearly greater than the decrease in Gγ synthesis, especially at later time points in the

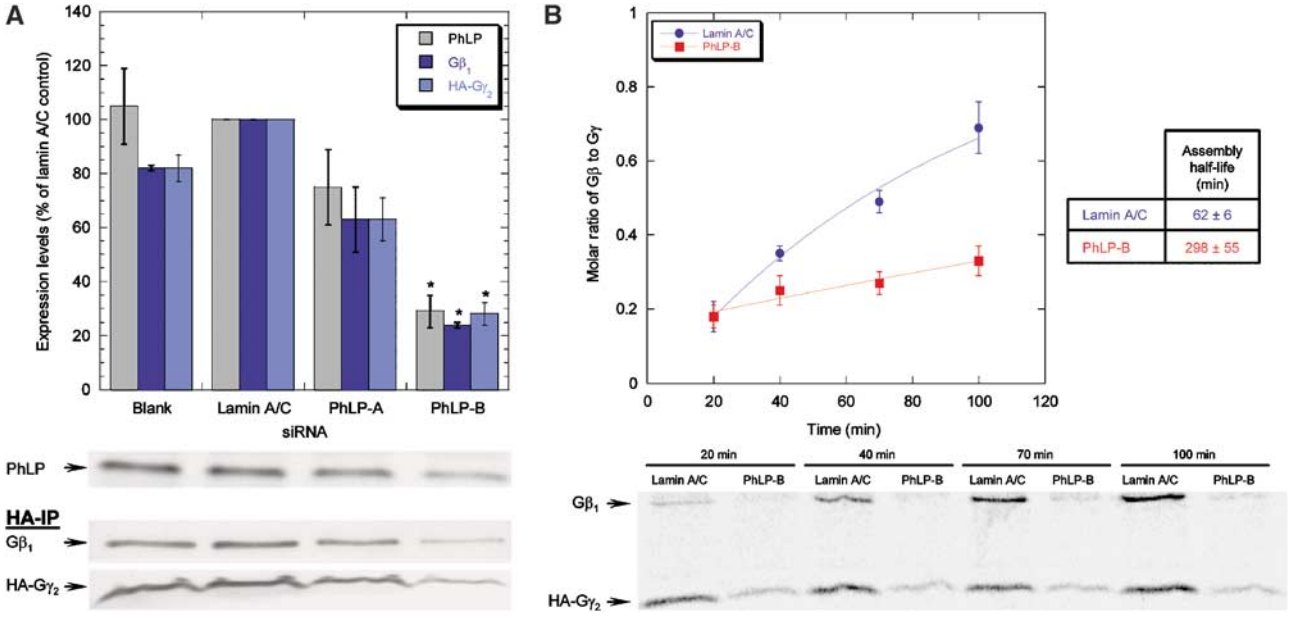

Figure 2 siRNA-mediated depletion of PhLP inhibits Gβγ dimer assembly. (**A**) HEK-293 cells were treated with the indicated siRNA and transiently transfected with $G\beta_1$ and HA-$G\gamma_2$ and then assayed after 96 h. Levels of overexpressed $G\beta_1\gamma_2$ dimer were determined by immunoprecipitating HA-$G\gamma_2$ and immunoblotting for $G\beta_1$ and HA-$G\gamma_2$. The effect of siRNA treatment on PhLP expression in HEK-293 cells was determined by immunoblotting whole-cell extracts for PhLP. Band intensities were quantified and expressed as a percentage of the lamin A/C sample. Bars and symbols in each panel represent the average ± s.e. from three or four separate experiments ($^*P<0.01$). (**B**) The rate of nascent $G\beta_1\gamma_2$ dimer formation was determined using a radiolabel pulse–chase assay. Cells were pulsed for 10 min with [^{35}S]methionine followed by a chase with unlabeled methionine. Times indicate the sum of the pulse and chase periods. After the chase, $G\beta_1\gamma_2$ dimers were immunoprecipitated with an antibody to HA-$G\gamma_2$. The dimers were resolved by Tris–Tricine–SDS–PAGE and radioactive protein bands were detected using a phosphorimager. Band intensities were quantified and molar ratios of $G\beta_1$ to $G\gamma_2$ were calculated. Lines represent a nonlinear least-squares fit of the data to a first-order rate equation. Values for $t_{1/2}$ are shown next to the graph.

chase period, suggesting that the rate of Gβγ assembly was also impaired in PhLP-depleted cells. To better assess this finding, the molar ratio of Gβ to Gγ was calculated at each time point during the chase period. A plot of the change in Gβ/Gγ ratio with time (Figure 2B) showed a significant decrease in the rate of assembly of Gβγ in PhLP-B siRNA-treated cells. The half-life for assembly of Gβγ in PhLP-depleted cells was ~300 min compared to ~60 min in lamin A/C siRNA-treated control cells. Similar results were observed when Gβ was immunoprecipitated. There was a two-fold reduction in Gβ synthesized and very little Gγ co-immunoprecipitated in PhLP-B siRNA-treated cells compared to the lamin A/C control (data not shown). Thus, these data support the idea that PhLP promotes the assembly of the Gβγ dimer.

PhLP phosphorylation at serines 18–20 is required for Gβγ dimer assembly

PhLP has been shown to be constitutively phosphorylated by CK2 at serines 18–20 (Humrich *et al*, 2003; Lukov *et al*, in preparation). Overexpression of a variant of PhLP in which these three residues were replaced by alanine (PhLP S18–20A) completely blocked the ability of overexpressed Gβγ to activate PLCβ in HEK-293 cells (Humrich *et al*, 2003). Subsequent experiments have shown that CK2 phosphorylation does not change the binding affinity of PhLP for Gβγ, but it does increase PhLP binding to CCT by three-fold (Lukov *et al*, in preparation). Coupling these findings with the observation that PhLP is required for Gβγ dimer assembly points to a role for CK2 phosphorylation in the regulation of Gβγ folding. To investigate this possibility, the effects of coexpression of PhLP S18–20A on Gβγ expression were measured by co-immunoprecipitation as in Figure 2A. The PhLP S18–20A variant inhibited Gβγ expression by approximately 70% compared to wild-type PhLP, while the empty vector control consistently showed 25% less Gβγ than wild-type PhLP (Figure 3A). This decrease in Gβγ expression was not attributable to a decrease in mRNA levels because Northern blot analyses showed no significant changes in overexpressed Gβ and Gγ mRNA when PhLP S18–20A was coexpressed (data not shown). Moreover, the effects of PhLP S18–20A overexpression appear to be a direct result of an inability to phosphorylate this site and were not caused by the alanine substitutions themselves because in the absence of CK2 phosphorylation, these substitutions had no effect on Gβγ or CCT binding (Lukov *et al*, in preparation).

To further explore the role of CK2 phosphorylation, the effects of overexpression of the PhLP S18–20A variant on Gγ translation and Gβγ assembly were measured in the pulse–chase experimental format. Overexpression of wild-type PhLP increased the rate of Gβγ assembly substantially when compared to the empty vector control. The $t_{1/2}$ for assembly was 12 min compared to 45 min for the control, nearly a four-fold increase (Figure 3B). In contrast, overexpression of the PhLP S18–20A variant caused a dramatic decrease in the rate of Gβγ assembly with a $t_{1/2}$ of ~180 min, more than 15-fold less than that of wild-type PhLP. The effects on Gγ translation were also very different. Wild-type PhLP had no effect, while PhLP S18–20A inhibited translation by 40%, similar to the decrease caused by PhLP depletion. These data confirm the role of PhLP as a positive regulator of Gβγ assembly and they show that CK2 phosphorylation at S18–20 is required for normal Gγ translation and Gβγ dimer assembly. Moreover, it appears that the overexpressed PhLP S18–20A interferes in some way with endogenous PhLP in performing these functions.

High-affinity binding of PhLP to Gβγ but not to CCT is necessary for Gβγ assembly

The findings that PhLP phosphorylation is necessary for Gβγ assembly and that phosphorylation increases the binding affinity of PhLP for CCT (Lukov *et al*, in preparation) suggest that the interaction between PhLP and CCT is necessary for assembly. This observation led to an analysis of the contribu-

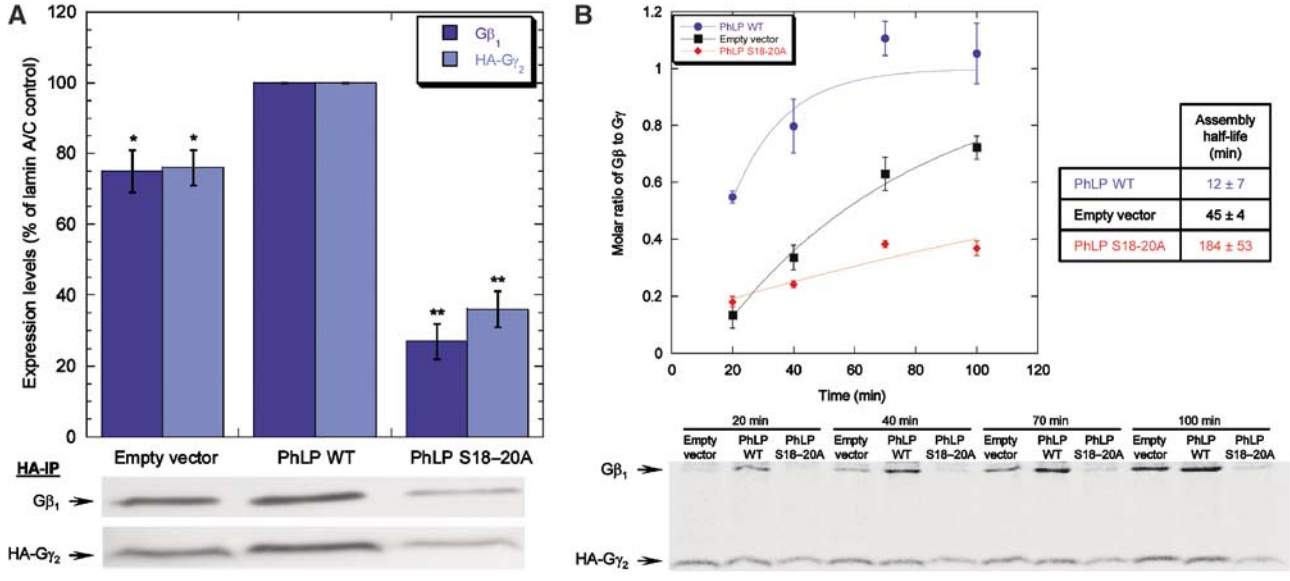

Figure 3 PhLP phosphorylation at serines 18–20 is required for Gβγ dimer assembly. (**A**) HEK-293 cells were transiently transfected with PhLP-myc, PhLP S18-20A or empty vector as indicated along with $G\beta_1$ and HA-$G\gamma_2$ and then assayed after 48 h. Levels of overexpressed $G\beta_1\gamma_2$ dimer were determined by immunoprecipitation as in Figure 2A. Bars represent the average ± s.e. from six separate experiments (*$P<0.01$, **$P<0.001$). (**B**) The rate of nascent $G\beta_1\gamma_2$ dimer formation was determined by a pulse–chase assay as in Figure 2B. Symbols represent the average ± s.e. from three separate experiments.

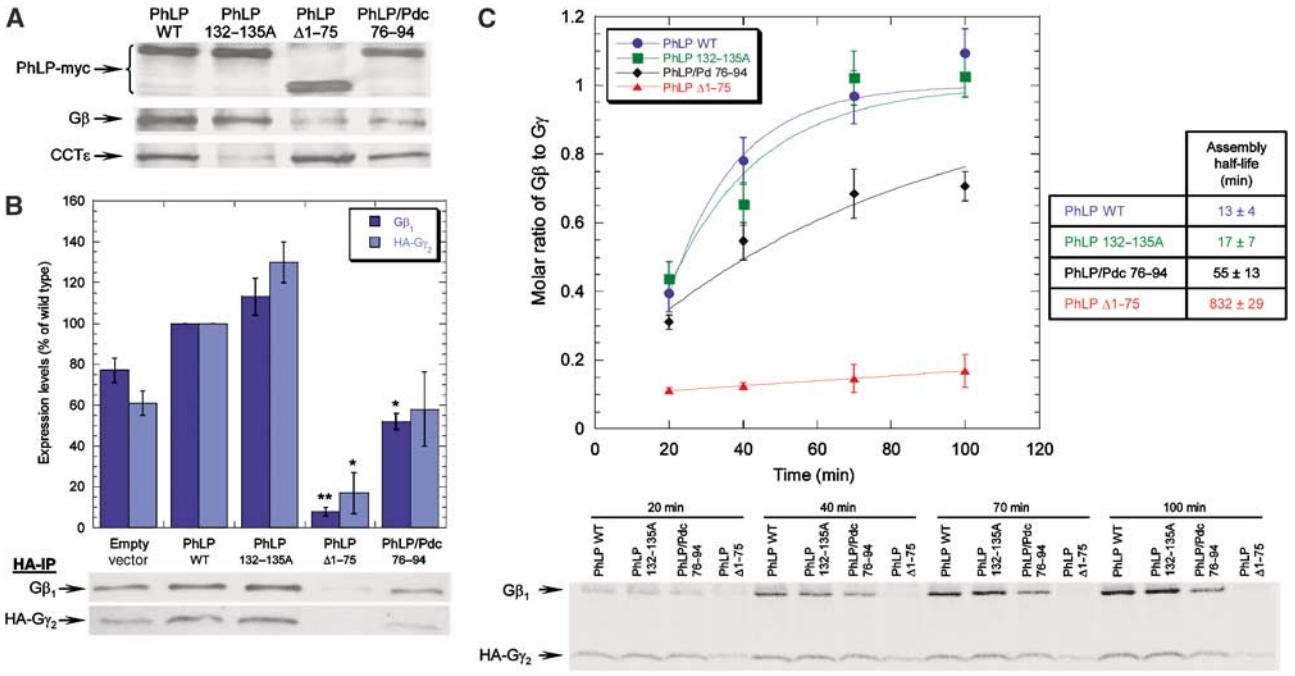

Figure 4 Interaction of PhLP with Gβγ but not with CCT is necessary for Gβγ dimer assembly. (**A**) HEK-293 cells were transiently transfected with PhLP-myc, PhLP 132–135A, PhLP Δ1–75, PhLP/Pdc 76–94, or empty vector as indicated along with $G\beta_1$ and HA-$G\gamma_2$ and then assayed after 48 h. The binding of PhLP variants to Gβγ and CCT was measured by immunoprecipitating with an antibody to the C-terminal myc tag and immunoblotting for PhLP, Gβ or CCTε. Blots are representative of similar data from three experiments. (**B**) Levels of overexpressed $G\beta_1\gamma_2$ dimer were determined by immunoprecipitation as in Figure 2A. Bars represent the average ± s.e. from three separate experiments ($^*P<0.01$, $^{**}P<0.001$). (**C**) The rate of nascent $G\beta_1\gamma_2$ dimer formation was determined by a pulse–chase assay as in Figure 2B. Symbols represent the average ± s.e. from three separate experiments.

tion of the PhLP–CCT interaction in Gβγ folding. A variant of PhLP with a greatly reduced binding affinity for CCT was prepared by substituting residues D_{132}DEE with alanine. These residues have been shown to contribute considerably to CCT binding (Martín-Benito *et al*, 2004). This PhLP 132–135A variant had normal Gβγ binding properties, but it bound CCT poorly in co-immunoprecipitation experiments from cells overexpressing the variant (Figure 4A). In addition, two other variants were prepared that bound CCT normally, but had reduced binding to Gβγ. The first was a truncation of PhLP in which residues 1–75 were deleted (PhLP Δ1–75). This variant lacks Helix 1, which is known to make a substantial contribution to Gβγ binding (Gaudet *et al*, 1996), yet it retains regions known to interact with CCT (Martín-Benito *et al*, 2004). The second variant was a chimera in which residues 76–94 of PhLP were replaced with Pdc sequence. Previous binding experiments showed that this PhLP/Pdc 76–94 variant had reduced Gβγ binding but normal CCT binding (Martín-Benito *et al*, 2004). As expected, PhLP Δ1–75 bound Gβγ poorly while PhLP/Pdc 76–94 showed intermediate binding, significantly less than wild-type PhLP yet more than PhLP Δ1–75 (Figure 4A). Both of these variants bound CCT normally (Figure 4A).

The effects of coexpression of these PhLP variants on cellular levels of overexpressed Gβγ were measured as in Figure 2A. Surprisingly, coexpression of PhLP 132–135A enhanced expression of Gβγ by 20% compared to wild-type PhLP, while the opposite effect was observed with coexpression of PhLP Δ1–75, which dramatically blocked Gβγ expression by 90% (Figure 4B). PhLP/Pdc 76–94 coexpression also inhibited Gβγ expression but the effect was less striking, about 50% less than wild type. Northern blot analysis showed the mRNA levels of overexpressed Gβ and Gγ in these cells were the same as that found in cells coexpressing wild-type PhLP (data not shown), indicating that inhibition of Gβγ expression in cells coexpressing PhLP Δ1–75 or PhLP/Pdc 76–94 was not caused by decreases in mRNA levels.

The effects of coexpression of these PhLP variants on Gγ translation and Gβγ assembly were also measured. PhLP 132–135A coexpression did not change the rate of Gγ translation and Gβγ dimer assembly when compared to wild-type PhLP (Figure 4C). On the other hand, the effect of PhLP Δ1–75 coexpression was striking, inhibiting translation of Gγ by 40% and completely blocking assembly. The effects of PhLP/Pdc 76–94 coexpression were more moderate, showing no effect on Gγ translation and reducing the rate of dimer assembly by four-fold. These results demonstrate that an interaction of PhLP with Gβγ is vital for assembly of the Gβγ dimer, and they suggest that high-affinity binding of PhLP to CCT is not necessary when PhLP is overexpressed.

PhLP associates with the nascent Gβ polypeptide in the absence of Gγ

To address the mechanism by which PhLP controls Gβγ assembly, the effects of combinatorial overexpression of each of the three G protein subunits on the ability of PhLP to form complexes with nascent Gβ or Gγ was assessed. PhLP was coexpressed with either $G\beta_1$ alone, $G\gamma_2$ alone, $G\beta_1$ and $G\gamma_2$ together or all three G protein subunits ($G\alpha_{i3}$, $G\beta_1$ and $G\gamma_2$) in HEK-293 cells, and the cells were pulsed with [^{35}S]methionine for 30 min to label the nascent polypeptides. Complexes of newly synthesized proteins associated with

PhLP, Gβ or Gγ were determined by co-immunoprecipitation. When all three G protein subunits were coexpressed together and PhLP was immunoprecipitated with an antibody to its C-terminal c-myc tag, significant amounts of nascent Gβ were found in the co-immunoprecipitate, but there was no nascent Gα or Gγ (Figure 5A, left four lanes). Gα was not expected to co-immunoprecipitate because it is known that Gα and PhLP compete for the same binding site on Gβ (Gaudet *et al*, 1996). However, it was very surprising not to find Gγ in the co-immunoprecipitate because PhLP has been shown to bind the Gβγ complex with moderately high affinity (Savage *et al*, 2000) and Gβ forms a very high-affinity complex with Gγ (Clapham and Neer, 1997). Similar results were seen when Gβ and Gγ or when Gβ alone were coexpressed, Gβ co-immunoprecipitated with PhLP without any detectible Gγ. Thus, the unanticipated conclusion from these data is that PhLP forms a complex with nascent Gβ in the absence of Gγ.

A similar conclusion can be made from Gγ immunoprecipitation experiments. The same cell extracts were immunoprecipitated with an antibody to the HA-tag on the

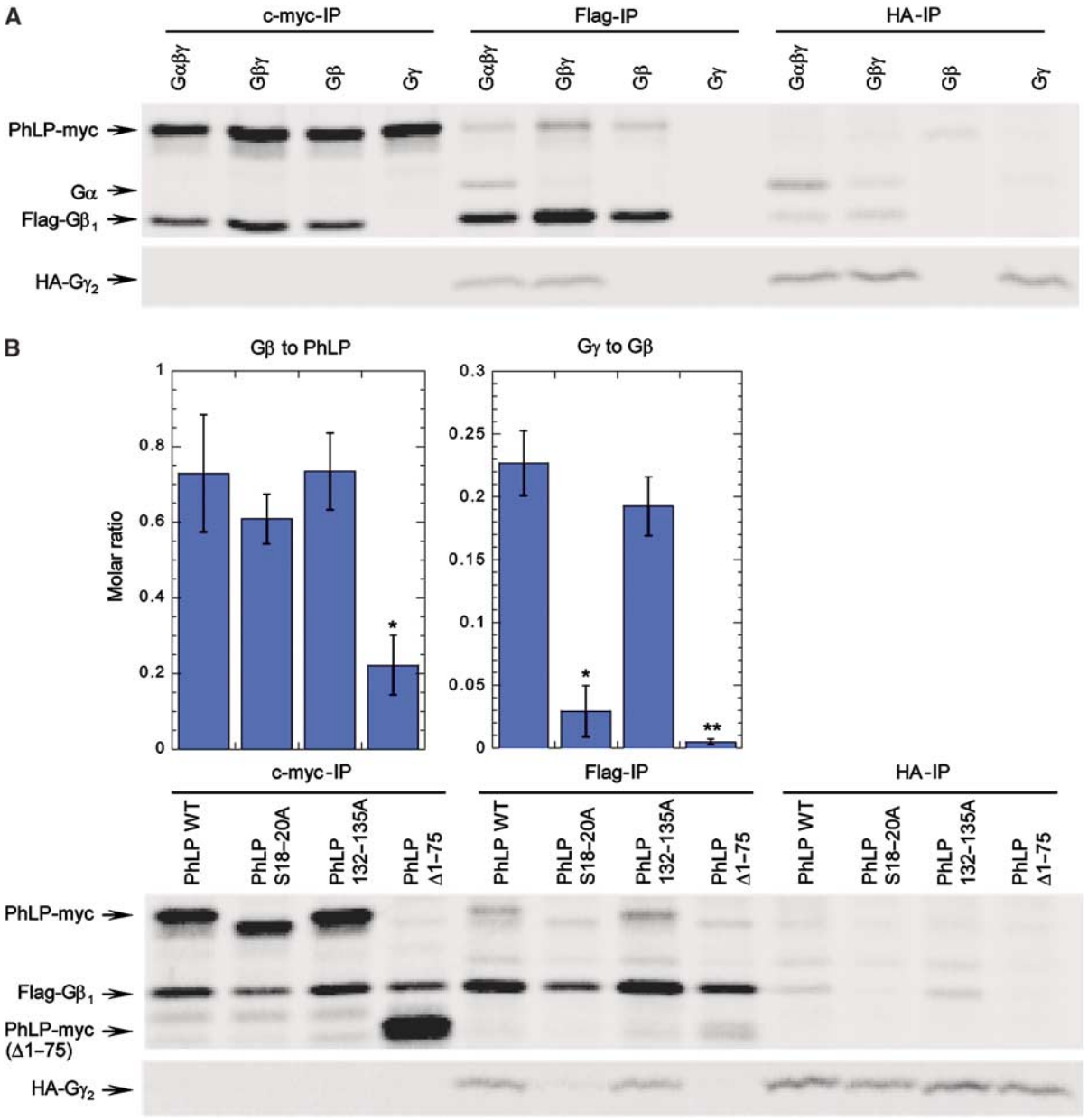

Figure 5 PhLP binds nascent Gβ in the absence of Gγ and requires S18–20 phosphorylation for Gγ to associate with the complex. (**A**) HEK-293 cells were transiently cotransfected with wild-type PhLP-myc in combination with $G\alpha_{i3}$, Flag-$G\beta_1$ and/or HA-$G\gamma_2$ as indicated. After 48 h, the cells were pulsed with [^{35}S]methionine for 30 min and extracts were immunoprecipitated using antibodies to the PhLP-myc, Flag-$G\beta_1$ or HA-$G\gamma_2$ epitope tags. The co-immunoprecipitating proteins were resolved on 10% Tris–Glycine–SDS gels for PhLP, Gα and Gβ and 16.5% Tris–Tricine–SDS gels for Gγ. Radioactive protein bands were detected using the phosphorimager. The gel shown is representative of similar gels from three separate experiments. (**B**) HEK-293 cells were transiently transfected with PhLP-myc, PhLP S18–20A, PhLP 132–135A or PhLP Δ1–75 as indicated along with Flag-$G\beta_1$- and HA-$G\gamma_2$-expressing vectors. After 48 h, the cells were pulsed with [^{35}S]methionine for 30 min, extracts were immunoprecipitated and were analyzed as in panel A. Band intensities were quantified and the indicated molar ratios of binding partners were calculated as in Figure 2B. A representative gel is shown. Bar graphs represent the average molar ratios ± s.e. from three separate experiments (*$P<0.01$, **$P<0.001$).

N-terminus of Gγ, resulting in co-immunoprecipitation of nascent Gβ and Gα, but not PhLP (Figure 5A, right four lanes). There was a minor band migrating just below the PhLP band that was observed in variable amounts in each of the four samples. This was a nonspecifically co-immunoprecipitating band since it was found in the sample expressing Gβ alone, which should have had no immunoprecipitate. When Gβ was immunoprecipitated from these same cell extracts, nascent PhLP, Gα and Gγ were co-immunoprecipitated whenever they were coexpressed (Figure 5A, middle four lanes), indicating that Gβ is in complexes with all three simultaneously. The composition of these complexes appears to be PhLP-Gβ, -Gβγ and -Gαβγ.

PhLP phosphorylation at S18–20 is needed for Gγ to associate with the PhLP-Gβ complex

To determine the role of CK2 phosphorylation, CCT binding and Gβγ binding in the assembly process, the PhLP variants deficient in these properties (PhLP S18–20A, PhLP 132–135A and PhLP Δ1–75, respectively) were coexpressed with Gβ and Gγ and their binding was measured as in Figure 5A. PhLP immunoprecipitation brought down detectible amounts of nascent Gβ in all of the samples (Figure 5B). However, plotting the ratio of Gβ to PhLP in the immunoprecipitates showed that there was significantly less Gβ relative to PhLP in the PhLP Δ1–75 sample, as expected from the decreased Gβγ binding of this variant. As observed in Figure 5A, there was no Gγ in any of the PhLP immunoprecipitates, confirming the observation that PhLP bound nascent Gβ in the absence of Gγ. In the Gβ immunoprecipitates, there was 60% less Gβ in the PhLP S18–20A or PhLP Δ1–75 samples compared to the wild type or PhLP 132–135A. The magnitude of this decrease cannot be attributed to a decrease in Gβ mRNA, so it appears that the decrease results from inhibition of Gβ translation in the presence of PhLP S18–20A or PhLP Δ1–75. Interestingly, there was no nascent Gγ in the Gβ immunoprecipitate when PhLP S18–20A and PhLP Δ1–75 were coexpressed, while Gγ was easily detected when PhLP and PhLP 132–135A were coexpressed. A plot of the Gγ to Gβ ratio showed that the lack of Gγ in the PhLP S18–20A and PhLP Δ1–75 samples was not merely a result of the decrease in Gβ expression, but was caused by a total inability of the nascent Gγ to associate with the Gβ in the presence of these PhLP variants. Consistent with this observation, there was no nascent Gβ in the Gγ immunoprecipitates when PhLP S18–20A and PhLP Δ1–75 were coexpressed, but there were detectible amounts of Gβ when wild type or PhLP 132–135A were coexpressed. There was also a 50% reduction in Gγ amounts when PhLP S18–20A and PhLP Δ1–75 were coexpressed, similar to what was observed in Figures 3B and 4C. As with Gβ, this reduction was not caused by a decrease in Gγ mRNA, hence it must have resulted from an inhibition of Gγ translation. The striking finding from these data is that phosphorylation of PhLP at S18–20 is required for Gβγ assembly to occur. Furthermore, the results show that an interaction between PhLP and Gβ is vital in the assembly process, while high-affinity binding to CCT is not.

Discussion

A new model for PhLP function

The observation that PhLP is a molecular chaperone for Gβγ assembly explains two opposing data sets regarding PhLP function that were heretofore irreconcilable. The first data set depicted PhLP as a negative regulator of G protein signaling by virtue of its ability to bind and sequester Gβγ from Gα and effectors by interacting with the same face of Gβ as all other Gβγ binding partners (Ford *et al*, 1998). This sequestration hypothesis was based on many studies in which exogenous PhLP or Pdc was either added to reconstituted systems or was overexpressed, resulting in inhibition of G protein signaling (McLaughlin *et al*, 2002a). However, endogenous PhLP concentrations were an order of magnitude less than those of Gβγ and supraphysiological concentrations were needed for inhibition to occur (McLaughlin *et al*, 2002a). The second data set is more recent and comes from genetic studies in which the *PhLP* gene was deleted (Kasahara *et al*, 2000; Garzon *et al*, 2002; Blaauw *et al*, 2003). PhLP deletion blocked G protein signaling (Blaauw *et al*, 2003), the opposite effect of that predicted by the sequestration hypothesis. These experiments suggested that PhLP was an essential, positive regulator of G protein signaling. The current study resolves this issue and confirms that PhLP is indeed essential for G protein signaling by virtue of its ability to catalyze the formation of Gβγ dimers. Without PhLP, the cell cannot assemble Gβγ and thus G protein signaling is blocked. The previous data on which the sequestration hypothesis was based can all be explained by the ability of excess PhLP to bind Gβγ and displace Gα or effectors, a process that does not occur at normal cellular expression levels where PhLP is limiting.

The findings reported here allow a model for the physiological function of PhLP in Gβγ assembly to be forwarded (Figure 6). PhLP appears to bind the nascent Gβ polypeptide early in the folding process and assist in the formation of its seven-bladed, β-propeller structure. The PhLP-Gβ complex is stable, allowing time for Gγ to associate with Gβ. The structure of the Pdc-Gβγ complex (Gaudet *et al*, 1996) gives insight into how Gγ might associate with PhLP-Gβ. Pdc binds Gβ on the interaction face and side of the β-propeller, opposite from the site of Gγ binding and making no contact with Gγ. By analogy, PhLP may hold Gβ in the proper conformation on one side while Gγ binds to the opposite side. In the absence of PhLP, Gβ is probably not able to fold into its β-propeller structure, making association with Gγ improbable. Once the PhLP-Gβγ complex is formed, one would expect PhLP to be displaced as Gβγ associates with the endoplasmic reticulum membrane and/or Gα (Michaelson *et al*, 2002). This prediction stems from the direct overlap of the binding footprint of PhLP on Gβγ with the membrane and Gα binding sites (Gaudet *et al*, 1996) and the resulting competition for Gβγ binding (Yoshida *et al*, 1994; Savage *et al*, 2000). Given the large membrane surface area of the endoplasmic reticulum and the excess of cellular Gα compared to PhLP (McLaughlin *et al*, 2002a), one would predict that the PhLP-Gβγ complex would exist only transiently. PhLP would be rapidly displaced from Gβγ, freeing it for another round of Gβγ assembly while the membrane-bound Gαβγ heterotrimer would be transported to the plasma membrane for activation by receptors (Michaelson *et al*, 2002). In this manner, PhLP would act catalytically to assemble Gβγ dimers, a function that is consistent with its lower level of expression compared to that of Gβ (McLaughlin *et al*, 2002a).

While the current work was under review, another report was published showing a requirement for PhLP in Gβγ dimer folding (Humrich *et al*, 2005). In this report, a model was

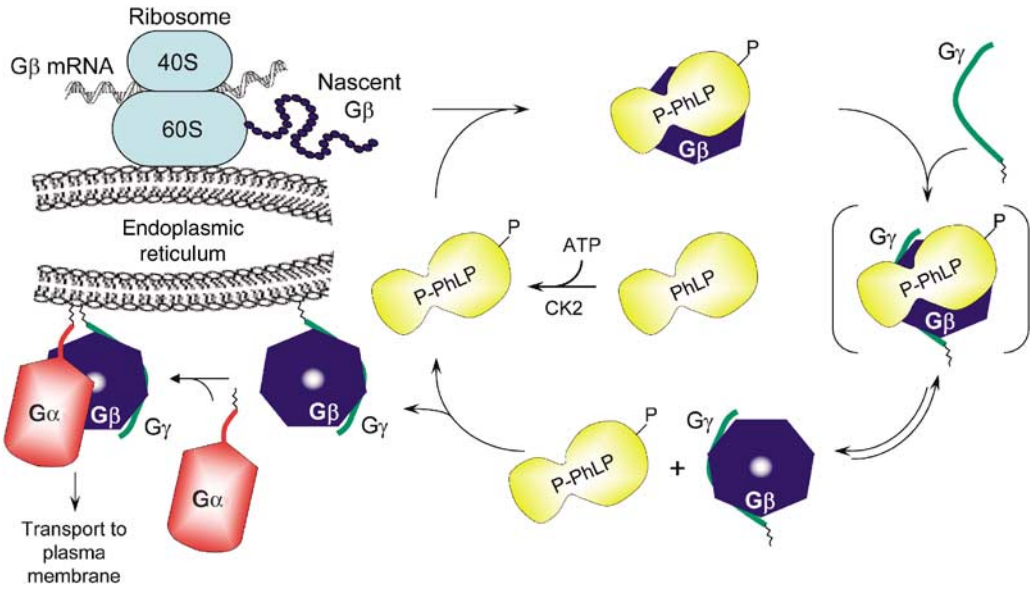

Figure 6 A model of PhLP-mediated Gβγ dimer assembly. The schematic diagram depicts a proposed mechanism for Gβγ dimer formation that is consistent with current data. CK2 phosphorylated PhLP binds and stabilizes the nascent Gβ polypeptide in a near-native conformation until Gγ can associate. PhLP-Gβγ forms a transient complex whose equilibrium is rapidly drawn toward dissociation because of Gβγ binding to the endoplasmic reticular membrane and Gα. See text for details.

proposed in which unphosphorylated PhLP would bind CCT and inhibit Gβγ assembly. However, this model is not consistent with the current observations that PhLP depletion blocked Gβγ assembly or that PhLP overexpression enhanced Gβγ assembly. These data require that PhLP play a positive role in Gβγ dimer formation.

Possible roles for CK2 phosphorylation of PhLP in Gβγ assembly

The results of Figures 3B and 5B show that CK2 phosphorylation of PhLP at serines 18–20 is essential for Gγ to associate with the PhLP-Gβ complex. This finding explains recent studies in which overexpression of two PhLP variants lacking the S18–20 phosphorylation site (PhLP S18–20A and PhLP Δ1–28) blocked the activation of PLCβ induced by overexpression of Gβγ to a much greater extent than wild-type PhLP (Humrich *et al*, 2003). These data could not be explained by the Gβγ sequestration hypothesis, but they are readily explained by the lack of Gβγ assembly caused by these PhLP variants. The mechanism by which S18–20 phosphorylation facilitates Gβγ dimer formation is not clear from the current data. Phosphorylation of serines 18–20 is not necessary for PhLP to interact with Gβ (Figure 5B), but perhaps phosphorylation induces a conformation in the PhLP-Gβ complex that is required for Gγ to associate.

An alternative mechanism would involve recruitment of another binding partner upon PhLP phosphorylation. The fact that phosphorylation of serines 18–20 increases PhLP binding to CCT (Lukov *et al*, in preparation) points to CCT as such a binding partner. However, the PhLP 132–135 variant with greatly reduced binding to CCT was as effective as wild-type PhLP in Gβγ assembly (Figures 4C and 5B), suggesting that high-affinity binding of PhLP to CCT is not necessary for Gβγ assembly. An interesting observation in this regard is that PhLP S18–20A and PhLP Δ1–75 variants inhibited the rate of Gβγ assembly to well below that of cells not over-expressing PhLP (Figures 3B and 4C), indicating that these variants were interfering with the ability of endogenous PhLP to catalyze Gβγ assembly. The dominant-negative effect of overexpressed PhLP S18–20A can be explained by its ability to displace endogenous PhLP from nascent Gβ, yielding a complex containing unphosphorylated PhLP, which may not be able to acquire a conformation that permits Gγ binding. PhLP Δ1–75 on the other hand would not be expected to displace endogenous PhLP from Gβ, but it might displace it from a necessary binding partner.

PhLP enhances Gβ and Gγ translation

In cells depleted of PhLP or in cells overexpressing PhLP S18–20A and PhLP Δ1–75 variants, there was a two-fold reduction in the amount of nascent Gβ and Gγ produced during [^{35}S]methionine pulses (Figures 2–5). This reduction appears to be attributable to a decrease in Gβ and Gγ translation because these treatments had little effect on Gβ and Gγ mRNA levels and because proteolytic degradation of monomeric Gβ and Gγ was not observed over the 100 min time course of these experiments (Figures 2B, 3B and 4C). Furthermore, the proteasome inhibitor MG-132 had no effect on the amount of nascent Gβ and Gγ produced during the 10 min pulse period (data not shown). Therefore, the only remaining explanation is that the decreases in nascent Gβ and Gγ expression come from reduced translation of their respective mRNAs. Perhaps, unassembled PhLP-Gβ or -Gγ inhibits further translation of the Gβ and Gγ mRNAs. Such a mechanism of assembly controlled translational regulation of the subunits of protein complexes has been described in other systems (Choquet *et al*, 2001).

Generality of PhLP-mediated Gβγ assembly among gene family members

A question yet to be addressed is whether other Pdc family members act as chaperones for Gβγ assembly in addition to

PhLP or whether they assist in the folding of other proteins with WD-40 β-propeller structures like Gβ. Pdc binds Gβγ and thus might assist in the formation of the $G\beta_1\gamma_1$ dimer, which is highly expressed along with Pdc in photoreceptor and pineal cells. The other Pdc family members PhLP2 and PhLP3 (Blaauw *et al*, 2003) bind Gβγ poorly (Flanary *et al*, 2000), suggesting that they are not involved in Gβγ assembly. However, genetic deletion of PhLP2 was lethal in both yeast and *Dictyostelium* (Flanary *et al*, 2000; Blaauw *et al*, 2003). In *Dictyostelium*, population doubling stopped after just a few days in *phlp2*$^-$ cells (Blaauw *et al*, 2003), indicating that perhaps PhLP2 participates in the folding of proteins vital for cell division. In this regard, PhLP2 in yeast forms a complex with CCT and VID27 (Aloy *et al*, 2004), a protein with WD-40 domain similar to Gβ. VID27 is believed to be involved in vacuolar protein degradation (Regelmann *et al*, 2003), but it may represent a class of WD40 domain-containing proteins that require PhLP2 and CCT for their folding (Valpuesta *et al*, 2002). Many of these WD40 proteins play a role in the progression of the cell cycle (Camasses *et al*, 2003), and if they were unable to achieve their native conformation in the absence of PhLP2, cell division would be blocked. In the case of PhLP3, its deletion is not lethal (Flanary *et al*, 2000; Blaauw *et al*, 2003), but there is genetic evidence that it is required for β-tubulin folding (Lacefield and Solomon, 2003). Thus, the Pdc gene family may be involved in the folding of several classes of protein folds.

In summary, these findings describe a novel physiological role for PhLP as an essential chaperone in Gβγ dimer formation and they give new insight into this essential process in G protein signaling. In a practical vein, these results indicate that coexpression of PhLP could increase yields of Gβγ, which are notoriously low in overexpression and purification protocols, and they suggest that PhLP and its phosphorylation by CK2 could be useful therapeutic targets to control G protein signaling in a general manner.

Materials and methods

Cell culture

HeLa S3 and HEK-293 cells were cultured in DMEM/F-12 (50/50 mix) growth media with L-glutamine and 15 mM HEPES, supplemented with 10% fetal bovine serum (HyClone). The cells were subcultured regularly in order to maintain active growth, but were not used beyond 15 passages.

RNA interference

siRNAs were chemically synthesized to target nucleotides 608–628 of human lamin A/C and nucleotides 152–172 and 345–365 of human PhLP. The PhLP siRNAs were designated PhLP-A and PhLP-B, respectively. The siRNA transfections were carried out as described previously (Elbashir *et al*, 2001). Briefly, HeLa S3 or HEK-293 cells were cultured in 24-well plates to 50–70% confluency. The cells were then transfected with siRNA at final concentration of 100 nM using Oligofectamine (Invitrogen). After 96 h, the cells were either harvested in 2% SDS for expression studies or used in subsequent assays. Protein levels were determined by immunoblotting as described previously (McLaughlin *et al*, 2002b). Briefly, cell lysates containing equal amounts of total protein (3–7 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and immunoblotted with polyclonal antibodies to PhLP (Thulin *et al*, 1999) or $G\beta_1$ (Lukov *et al*, 2004), or a monoclonal antibody to lamin A/C (Santa Cruz Biotechnology).

In vivo Ca^{2+} measurement

HeLa S3 cells were treated for 48 h with siRNA and then subcultured into clear-bottomed, black-sided 96-well plates and allowed to recover for 24 h. The cells were then washed and serum starved in 100 μl of serum-free media for 18 h. After the starvation, 100 μl of Calcium Assay Plus Dye (Molecular Devices) with 1 mM Probenicide were added to the wells. At time zero, the cells were stimulated with 50 nM histamine and intracellular Ca^{2+} levels were measured using a FlexStation plate reader (Molecular Devices) by excitation at 480 nm and detection at 525 nm. Data were normalized to the baseline values before histamine treatment.

Preparation of cDNA constructs

Wild-type human PhLP with 3′ c-myc and His_6 epitope tags was constructed in pcDNA3.1/myc-His B vector as described (Carter *et al*, 2004). The PhLP Δ1–75 deletion variant was prepared by PCR amplification of the nucleotides corresponding to amino acids 76–301 and insertion in pcDNA3.1/myc-His B. A starting ATG sequence was placed in front of the PhLP fragment to ensure proper expression of the variant. The PhLP 132–135A variant was prepared by substituting D_{132}DEE for alanine codons, thereby creating a unique *Sac*II restriction site. Two PhLP fragments were amplified using the T7 forward primer from the vector with a PhLP reverse primer (5′-CAA GCC GCC GCG GCA TTT CTG CAG CAG TAC CGG AAG-3′) containing the *Sac*II site (CCGCGG) and a PhLP forward primer (5′-AAA TGC CGC GGC GGC TTG GTC CTC ATT CAT TAT GGC-3′) containing the *Sac*II site with a BGH reverse primer from the vector. The fragments were digested with *Sac*II, gel purified and ligated. The product was amplified using T7 forward and BGH reverse primers and inserted in pcDNA3.1/myc-His B. The PhLP/Pdc 76–94 chimera was prepared in pcDNA3.1/myc-His B using the same strategy as described previously (Martín-Benito *et al*, 2004). The CK2 site variant of PhLP (PhLP S18–20A) in which S_{18}SS were substituted for alanine codons was constructed in pcDNA3.1/myc-His B using the same method as with PhLP 132–135A. All constructs were inserted into the vector at the *Eco*RI and *Xba*I sites. The pcDNA3.1 vectors containing N-terminally HA-tagged $G\gamma_2$, untagged $G\beta_1$, N-terminally Flag-tagged $G\beta_1$ or $G\alpha_{i3}$ were obtained from the UMR cDNA Resource Center.

Transient transfections

HEK-293 cells were siRNA treated and then transfected 24 h later with 1.0 μg each of HA-$G\gamma_2$ and $G\beta_1$ in the pcDNA3.1 vector using Lipofectamine Plus Reagent according to the manufacturer's protocol (Invitrogen). The cells were used in subsequent applications 72 h later. For coexpression experiments not involving siRNA treatment, HEK-293 cells were plated in 24-well plates so that they were 70–80% confluent the next day. The cells were then transfected with 0.2 μg of either wild-type PhLP-myc, one of the PhLP-myc variants or the empty vector as a control along with 0.2 μg each of HA-$G\gamma_2$ and -$G\beta_1$. In experiments involving two to four cotransfections, the indicated combinations of PhLP-myc, HA-Gγ, Flag-Gβ or $G\alpha_{i3}$ cDNAs in pcDNA 3.1 were transfected 24 h after plating in six-well plates using 0.5 μg of each vector. The cells were harvested for subsequent applications 48 h after transfection.

Radiolabel pulse–chase assay

Transfected HEK-293 cells in 24-well plates were washed and incubated for 1 h in 500 μl of methionine-free DMEM media (Mediatech Inc.) supplemented with 4 mM L-glutamine (Sigma), 0.063 g/l L-cystine dihydrochloride (USB) and 10% dialyzed fetal bovine serum (Invitrogen). The media were discarded and 200 μl of new media supplemented with 200 μCi/ml radiolabeled L-[^{35}S]methionine (Amersham Biosciences) was added. The cells were then incubated at 23°C for 10 min to incorporate the [^{35}S]methionine into newly synthesized proteins. After this pulse phase, the cells were washed and incubated at 23°C for the time periods indicated in 1 ml of media supplemented with 4 mM nonradiaolabeled L-methionine (Sigma) to stop the [^{35}S]methionine incorporation. Following this chase period, the cells were harvested for immunoprecipitation experiments. When multiple immunoprecipitations were carried out from the same cell extract (Figure 5), a six-well plate format was used. The cells were washed and incubated in 2 ml of the methionine-free media, incubated for 30 min in 1 ml of the L-[^{35}S]methionine media, and then harvested for immunoprecipitation experiments.

Immunoprecipitation experiments

Transfected HEK-293 cells were washed with phosphate-buffered saline (PBS) (Fisher) and solubilized in immunoprecipitation buffer

(PBS, pH 7.4, 2% IGEPAL (Sigma), 0.6 mM PMSF, 1 μg/ml leupeptin, pepstatin and aprotinin). The lysates were passed 10 times through a 25 G needle and centrifuged at maximum speed for 10 min at 4°C in an Eppendorf microfuge. The clarified lysates were incubated with 2 μg of anti-c-myc (clone 9E10, BioMol), anti-HA (clone 3F10, Roche) or anti-Flag (clone M2, Sigma) antibodies and 25 μl of a 50% slurry of Protein A/G Plus agarose (Santa Cruz Biotechnology) as described previously (McLaughlin *et al*, 2002b). The precipitate was solubilized in SDS sample buffer and resolved on 10% Tris–Glycine–SDS or 16.5% Tris–Tricine–SDS gels. The gels were either dried for radioactivity measurements, or they were immunoblotted using the $G\beta_1$, c-myc or HA antibodies described above, or a rabbit polyclonal anti-CCTε antibody (Martín-Benito *et al*, 2004). Immunoblots were developed with the ECL Plus chemiluminescence reagent (Amersham). Gels and immunoblots were visualized with a Storm 860 phosphorimager, and the band intensities were quantified using Image Quant software (GE Healthcare). The molar ratios were determined by normalizing the band intensities to the number of methionine residues found in PhLP, Gβ and Gγ and then calculating the ratios as indicated. The rate data for Gβγ assembly were fit to a first-order rate equation with background correction to determine the rate constant (k), and the $t_{1/2}$ for assembly was calculated as follows: $t_{1/2} = \ln 2/k$.

Northern blotting

The effects of siRNA treatment or PhLP variant overexpression on endogenous Gβ or overexpressed Gβ and Gγ mRNA levels were determined by Northern blotting. Total RNA was isolated from cells in a six-well plate using the RNAqueous kit (Ambion). RNA was loaded (15 μg/well) and separated on 1.0–1.5% agarose/8.0% formaldehyde gels. Gels were transferred to a Hybond-N^+ membrane (Amersham) by capillary action for 1 h in 0.01 M NaOH, 3 M NaCl for the endogenous Gβ mRNA or for 16 h in 10 × SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for the overexpressed Gβ and Gγ mRNAs. The membranes were prehybrized with ExpressHyb hybridization buffer (Clonetech) for 1 h at 65°C. A radiolabeled probe was prepared using a PCR amplicon from 500 bp of the 3′-untranslated region of the wild-type $G\beta_1$ gene as a template or from the entire coding region of the overexpressed $G\beta_1$ and $G\gamma_2$ cDNAs. The probe was radiolabeled by the random hexamer method using the Prime-a-Gene labeling system (Promega). Membranes were hybridized with the probe in ExpressHyb buffer for 1 h at 65°C and washed twice for 15 min at 37°C. Radioactivity was visualized and quantified using the phosphorimager. Membranes were then stripped by washing twice in 0.1% SDS for 15 min at 95°C and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The Gβ and Gγ band intensities were normalized to the GAPDH band intensity.

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