Mechanism of Assembly of G Protein βγ Subunits by Protein Kinase CK2-phosphorylated Phosducin-like Protein and the Cytosolic Chaperonin Complex

From the Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602

Phosducin-like protein (PhLP) is a widely expressed binding partner of the G protein βγ subunit complex (Gβγ) that has been recently shown to catalyze the formation of the Gβγ dimer from its nascent polypeptides. Phosphorylation of PhLP at one or more of three consecutive serines (Ser-18, Ser-19, and Ser-20) is necessary for Gβγ dimer formation and is believed to be mediated by the protein kinase CK2. Moreover, several lines of evidence suggest that the cytosolic chaperonin complex (CCT) may work in concert with PhLP in the Gβγ-assembly process. The results reported here delineate a mechanism for Gβγ assembly in which a stable ternary complex is formed between PhLP, the nascent Gβ subunit, and CCT that does not include Gγ. PhLP phosphorylation permits the release of a PhLP-Gβ intermediate from CCT, allowing Gγ to associate with Gβ in this intermediate complex. Subsequent interaction of Gβγ with membranes releases PhLP for another round of assembly.

Eukaryotic cells employ heterotrimeric G proteins to transduce a wide variety of hormonal, neuronal, and sensory signals that control numerous physiological processes. As a result, malfunctions in G protein pathways contribute to many diseases (1–3), and therapeutic agents targeting G protein-coupled receptors represent the fundamental steps in the propagation of a G protein-mediated signal. From the Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602.

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Georgi L. Lukov, Christine M. Baker, Paul J. Ludtke, Ting Hu, Michael D. Carter, Ryan A. Hackett, Craig D. Thulin, and Barry M. Willardson

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1 To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, C-100 BNSN, Brigham Young University, Provo, UT 84602. Tel.: 801-422-2785; Fax: 801-422-0153; E-mail: barry_willardson@byu.edu.

2 The abbreviations used are: Gα, α subunit of a heterotrimeric G protein; G, heterotrimeric GTP-binding protein; Pdc, phosducin; PhLP, phosducin-like protein; CCT, cytosolic chaperonin containing tailless complex polypeptide 1 (CCT), an essential molecular chaperone that mediates the folding of actin, tubulin, and other proteins into their native structures (17). 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 (18) 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 co-chaperone that binds nascent actin polypeptide chains and delivers them to CCT for folding (19). Coupling these observations with the fact that yeast Gβ (20) and other proteins with seven β-propeller structures similar to Gβ (21–23) interact with CCT suggests that PhLP might function as a co-chaperone in the folding of Gβ. Indeed, recent findings show that PhLP does act as an essential chaperone for assembly of the Gβγ dimer, demonstrating that the physiological function of PhLP is not to down-regulate G protein signaling by sequestering Gβγ. However, the results of several recent studies have seriously challenged this model. Specifically, disruption of the Phlp1 gene in the chestnut blight fungus Cryphonectria parasitica (15) and in the soil amoeba Dictyostelium discoideum (7) yielded the same phenotype as the disruption of the Gβ gene. Moreover, PhLP depletion blocked G protein signaling in Dictyostelium (7). 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 (16). All of these observations are the exact opposite of what would be predicted by the Gβγ sequestration model.

Insight into an alternative function of PhLP has come from the observation that PhLP interacts with the cytosolic chaperonin containing tailless complex polypeptide 1 (CCT), an essential molecular chaperone that mediates the folding of actin, tubulin, and other proteins into their native structures (17). 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 (18) 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 co-chaperone that binds nascent actin polypeptide chains and delivers them to CCT for folding (19). Coupling these observations with the fact that yeast Gβ (20) and other proteins with seven β-propeller structures similar to Gβ (21–23) interact with CCT suggests that PhLP might function as a co-chaperone in the folding of Gβ. Indeed, recent findings show that PhLP does act as an essential chaperone for assembly of the Gβγ dimer, demonstrating that the physiological function of PhLP is not to down-regulate G protein signaling by sequester-
ing Gβγ but to support G protein signaling by catalyzing Gβγ dimer formation (24). However, the role of CCT in this process remains unclear (24, 25).

Phosphorylation of PhLP by protein kinase CK2 (CK2) plays an important role in PhLP function. A major site of CK2 phosphorylation occurs within a sequence of three consecutive serines (residues 18–20) near the N terminus (14).Phosphorylation of serines 18–20 was required for PhLP-mediated Gβγ assembly, for when these residues were substituted with alanine, PhLP was unable to catalyze Gβγ dimer formation (24). The mechanism by which phosphorylation at these sites enhances Gβγ dimer formation is not known; therefore, the effects of CK2 phosphorylation on PhLP function were investigated. The results of these studies provide evidence for a mechanism of PhLP-mediated Gβγ assembly that involves the formation of a ternary complex between PhLP, the nascent Gβ polypeptide, and CCT. PhLP phosphorylation is required for the release of PhLP-Gβ from the CCT complex and the subsequent association of Gγ with Gβ to form the Gβγ dimer.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK-293 and CHO cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (50/50 mix) growth media with 1% glutamine and 15 mM HEPES, supplemented with 10% fetal bovine serum (HyClone). The cells were subcultured regularly to maintain active growth but were not used beyond 20–25 passages.

**Preparation of cDNA Constructs**—Wild-type human PhLP, PhLP Δ1–75, and Pdc with 3′c-myc and His6 tags in the pcDNA3.1/myc-His B vector (Invitrogen) were prepared as described (24). Serine-to-alanine variants of human PhLP at positions 18, 19, 20, 25, and 296 were constructed in the pcDNA3.1/myc-His B vector by employing a PCR-based strategy and utilizing unique endonuclease restriction sites near the substitution site as described (24). The constructs were then subcloned into the bacterial expression vector pET15b (Novagen) as described (26). The integrity of all constructs was confirmed by sequence analysis. The N-terminally hemagglutinin (HA)-tagged Gγ2, FLAG epitope-tagged Gβ1, and untagged Gβ1 cDNAs also in the pcDNA3.1 vector were obtained from the UMR cDNA Resource Center.

**Protein Expression and Purification**—Wild-type and CK2 phosphorylation site variants of human PhLP in the pET15b vector were transformed in *Escherichia coli* DE3 cells and were purified using nondenaturing Ni2+ affinity chromatography as described previously (11). The purified proteins were concentrated and were stored in 50% glycerol at −80°C. The phosphorylation site variants of human PhLP in the pET15b vector were prepared as described (24). The integrity of all constructs was confirmed by sequence analysis. The N-terminally hemagglutinin (HA)-tagged Gγ2, FLAG epitope-tagged Gβ1, and untagged Gβ1 cDNAs also in the pcDNA3.1 vector were obtained from the UMR cDNA Resource Center.

**Electrophoretic Mobility Determinations**—CHO cells were plated in 6-well plates so that they were 70–80% confluent the following day. They were then co-transfected with 1 μg of either wild-type PhLP-myc or one of the CK2 phosphorylation site variants using Lipofectamine Plus reagent according to the manufacturer’s protocol. The cells were harvested 48 h later in 200 μl of immunoprecipitation buffer (24), and the PhLP-myc was immunoprecipitated from the lysate with 3 μg of anti-c-myc antibody and 30 μl of Protein A/G beads as described previously (17, 24). The final precipitate was solubilized in 40 μl of 2× SDS-PAGE sample buffer, and 10 μl of each sample was resolved on 10% SDS-PAGE gels. The gels were immunoblotted with a 1:1000 dilution of the anti-c-myc antibody and developed as described above.

**Gβγ Expression Measurements**—HEK-293 cells were plated in 6-well plates so that they would be 70–80% confluent the following day. They were then co-transfected with 1 μg of each of the PhLP-myc, HA-Gγ2, and Gβ1 cDNAs using Lipofectamine Plus reagent. In all experiments involving multiple transfections, the total amount of cDNA was held constant by...
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adding empty vector. After 48 h, the cells were washed and solubilized in 200 μl of immunoprecipitation buffer. The Gβ1γ2 complexes were immunoprecipitated from 100 μl of the lysate with 1.5 μg of anti-HA (clone 3F10, Roche Applied Science) antibody as described previously (17, 24). The complexes were solubilized in 40 μl of 2 × SDS sample buffer, and 10 μl was resolved on 10% Tris-glycine SDS-PAGE gels for Gβ1γ2. For Gβ detection, the gels were immunoblotted with a 1:2000 dilution of anti-HA (clone 3F10, Roche Applied Science). The complexes were immunoprecipitated from 100 μl of the lysate and Gβγ are shown. Binding was measured by immunoprecipitation of PhLP coupled with detection of the co-immunoprecipitating CCT or Gβ by immunoblotting. A representative immunoblot is shown. The graph gives the average intensity ± S.E. of the CCT bands relative to the unphosphorylated sample from eight separate experiments. C, the effects of CK2 phosphorylation on the ability of PhLP to inhibit Gβ1γ2-assisted binding of 125I-labeled Gα to membranes containing light-activated rhodopsin were determined. The graph gives the average ± S.E. from three separate experiments.

Radiolabel Pulse-Chase Assay—The pulse-chase assay was performed and quantified as described previously (24). A similar protocol was used to measure the rate of release of nascent Gβ from CCT. Six-well plates of HEK-293 cells were co-transfected with 1.0 μg of FLAG-Gβ1, HA-Gγ2, or PhLP-myc cDNAs as indicated in Fig. 6A. After 24 h, cells were lysed and extracts were immunoprecipitated with 2.5 μg of anti-CCTε antibody (Sero-tec), the immunoprecipitates were resolved on SDS-PAGE gels and immunoblotted for FLAG-Gβ1, PhLP-myc, Pdc-myc, or HA-Gγ2 using the indicated antibodies as described above. Gβ1 bands were quantified as described above, and intensities were calculated as a percentage of the control as indicated.

For binding experiments involving endogenous Gβ, HEK-293 cells were grown in 100-mm dishes and transfected with 6.0 μg of PhLP variant cDNAs as indicated in Fig. 5B. Cells were lysed in 1.2 ml of buffer, and 1 ml was immunoprecipitated with 10 μg of anti-CCTε antibody and 60 μl of protein A/G beads. Endogenous Gβ1 was detected with the anti-Gβ1 antibody. For binding experiments involving endogenous PhLP, HEK-293 cells were grown in 6-well plates and transfected with 1.0 μg of FLAG-Gβ1 cDNA as indicated in Fig. 5C. Extracts from two wells were pooled, and 200 μl was immunoprecipitated with 3 μg of anti-CCTε antibody. Other immunoprecipitation and immunoblotting procedures were as described above.

RESULTS

Effects of CK2 Phosphorylation on PhLP on CCT and Gβγ Binding—To begin to assess the impact of CK2 phosphorylation on PhLP function, the effects of phosphorylation on the binding of PhLP to its two known binding partners, Gβγ and CCT, were determined in vitro. Purified recombinant human PhLP was readily phosphorylated by CK2, resulting in a marked reduction in the mobility of the PhLP protein band in SDS-PAGE gels (Fig. 1A). The entire PhLP band was shifted, indicating that phosphorylation was 100% complete under the conditions used. The effects of CK2 phosphorylation on CCT binding

**FIGURE 1. Effects of CK2 phosphorylation on PhLP binding to CCT and Gβγ**. A, the decrease in mobility of PhLP in SDS-PAGE upon CK2 phosphorylation is shown. PhLP was phosphorylated by CK2 in vitro (P-PhLP) and was analyzed on a 10% gel along with unphosphorylated PhLP (B). The effects of CK2 phosphorylation on the binding of PhLP to CCT and Gβγ are shown. Binding was measured by immunoprecipitation of PhLP coupled with detection of the co-immunoprecipitating CCTε or Gβ by immunoblotting. A representative immunoblot is shown. The graph gives the average intensity ± S.E. of the CCT bands relative to the unphosphorylated sample from eight separate experiments. C, the effects of CK2 phosphorylation on the ability of PhLP to inhibit Gβ1γ2-assisted binding of 125I-labeled Gα to membranes containing light-activated rhodopsin were determined. The graph gives the average ± S.E. from three separate experiments.

The pulse-chase assay was performed for the times indicated and the cells were harvested in 220 μl of immunoprecipitation buffer. The extract was divided into two 95-μl samples, and 2.5 μl of 1 μg/μl anti-CCTε antibody (Sero-tec) was added to one sample and 3.0 μl of 1 μg/μl anti-FLAG antibody was added to the other sample. The immunoprecipitation and analysis of the radiolabeled proteins co-immunoprecipitating with CCT were carried out as described (24). The Gβ1 band was clearly separated from the other radiolabeled bands, facilitating its quantification. The amount of Gβ1 in the CCT immunoprecipitate was divided by that in the FLAG-Gβ1 immunoprecipitate to determine the fraction of the total Gβ1 bound to CCT. These values were expressed as a percentage of the 30-min time point to readily compare the rates of Gβ dissociation from CCT. The data were fit to a first order dissociation rate equation using the KaleidaGraph graphics software to determine the dissociation rate constant k. From the k values, the half-life was calculated by the equation, \( t_{1/2} = \ln 2/k \).

Assay of Gβ Binding to CCT—For CCT binding experiments involving Gβ1 overexpression, HEK-293 cells were plated in 6-well plates and transfected with 1.0 μg of FLAG-Gβ1, HA-Gγ2, Pdc, or PhLP cDNAs as indicated in Fig. 5 (A and C). Alternatively, the transfections were performed with 0.5 μg of FLAG-Gβ1, 1.0 μg of PhLP variants, and 1.5 μg of HA-Gγ2 as indicated in Fig. 6A. After 48 h, cells were lysed and extracts were immunoprecipitated with 2.5 μg of anti-CCTε antibody (Sero-tec), the immunoprecipitates were resolved on SDS-PAGE gels and immunoblotted for FLAG-Gβ1, PhLP-myc, Pdc-myc, or HA-Gγ2 using the indicated antibodies as described above. Gβ1 bands were quantified as described above, and intensities were calculated as a percentage of the control as indicated.

For binding experiments involving endogenous Gβ, HEK-293 cells were grown in 100-mm dishes and transfected with 6.0 μg of PhLP variant cDNAs as indicated in Fig. 5B. Cells were lysed in 1.2 ml of buffer, and 1 ml was immunoprecipitated with 10 μg of anti-CCTε antibody and 60 μl of protein A/G beads. Endogenous Gβ1 was detected with the anti-Gβ1 antibody. For binding experiments involving endogenous PhLP, HEK-293 cells were grown in 6-well plates and transfected with 1.0 μg of FLAG-Gβ1 cDNA as indicated in Fig. 5C. Extracts from two wells were pooled, and 200 μl was immunoprecipitated with 3 μg of anti-CCTε antibody. Other immunoprecipitation and immunoblotting procedures were as described above.
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A

N28-\text{SATCHSED}_{30}^{\text{PO}_4} \text{DLEID}_{30}^{\text{ALEGPR}} \text{with one phosphate}

B

L13-QYYYSSEDEDSDHEDKDR_{32} \text{with one phosphate}

C

L13-QYYYSSEDEDSDHEDKDR_{32} \text{with two phosphates}
was assessed by measuring the ability of PhLP to co-immunoprecipitate CCT from rabbit reticulocyte lysate (18). Human PhLP bound CCT with a high affinity, as evidenced by the fact that addition of only 5 nM PhLP was sufficient to co-immunoprecipitate readily detectable amounts of CCT from the reticulocyte lysate. Under these conditions, CK2 phosphorylation increased the co-immunoprecipitation of CCT by 7-fold (Fig. 1B). In contrast, CK2 phosphorylation had no effect on the ability of PhLP to co-immunoprecipitate purified Gβγ1γ2 (Fig. 1B) or to inhibit association of Gβγ1γ2 with Gα and light-activated rhodopsin (Fig. 1C), indicating that phosphorylation did not change the binding of PhLP to Gβγ1γ2.

**Mass Spectrometric Analysis of the CK2 Phosphorylation Sites of PhLP**—The phosphorylation sites that could potentially be responsible for the increase in PhLP binding to CCT upon CK2 phosphorylation were identified by mass spectrometry. PhLP was phosphorylated by CK2 in vitro and digested with trypsin, and the resulting peptide fragments were analyzed by electrospray tandem mass spectrometry. Fig. 2A shows the collision-induced dissociation (CID) spectrum of a doubly charged parent ion with an m/z ratio of 1198.5, corresponding to the mass of a tryptic peptide containing the C-terminal residues 287–301 of PhLP plus one phosphate. This CID spectrum showed robust peaks for both the b and y ions corresponding to the sequence of the 287–301 peptide. Of these, the y16 ion had an m/z equal to the loss of a phosphate, and the formation of a dehydroalanine at one of the two serines in this fragment, indicating that either Ser-293 or Ser-296 was phosphorylated in the parent ion. The b2, b4, b6, and b9 ions all had m/z ratios corresponding to the mass of their unphosphorylated fragments, suggesting that Ser-288 and Ser-293 were not phosphorylated. Therefore, the phosphate most likely resided on Ser-296. Accordingly, Ser-296 is within a strong consensus site for CK2 phosphorylation with negatively charged residues at the +1 and +3 positions (28).

One other tryptic fragment with m/z values corresponding to a phosphorylated species was detected and analyzed by CID. This peptide consisted of PhLP residues 13–32 plus one and two phosphates. The spectrum for the singly phosphorylated species yielded few b and y ions, none of which were phosphorylated. However, there were sufficient fragments to confirm the identity of the peptide (Fig. 2B). The same result was obtained with the doubly phosphorylated species. The CID spectrum confirmed the identity of the peptide but did not show any phosphorylated fragments (Fig. 2C). Hence, the four serines of this peptide, serines 18–20 and 25, could all be considered as potential CK2 phosphorylation sites. Of the four, Ser-20 and Ser-25 are within CK2 consensus sites, and phosphorylation of Ser-20 would create a strong consensus site for CK2 phosphorylation of Ser-19 in a doubly phosphorylated species. Similarly, phosphorylation of Ser-19 would make Ser-18 a good CK2 site, although no triply phosphorylated species of the 18–32 peptide were detected. Together, the mass spectrometric data suggest five potential CK2 phosphorylation sites on PhLP: Ser-18, Ser-19, Ser-20, Ser-25, and Ser-296.

**Contribution of Specific CK2 Phosphorylation Sites to the PhLP-CCT Interaction**—To identify which of these sites is responsible for the phosphorylation-dependent increase in PhLP binding to CCT, each of the five serines identified above was substituted with alanine in various combinations. The resulting PhLP variants were CK2-phosphorylated, and their binding to CCT was determined as described in Fig. 1. Substitution of one residue within the serine 18–20 sequence caused only minor reductions in the phosphorylation-induced increase in binding, whereas substitution of two residues within this sequence resulted in reductions in the phosphorylation-induced binding from 7-fold to ~4-fold (Fig. 3). Replacement of all three serines within this phosphorylation site caused a further reduction in the phosphorylation-induced increase to 3-fold, indicating that multiple phosphorylation events within the serine 18–20 site were responsible for much of the observed increase in PhLP binding to CCT upon CK2 phosphorylation.

With regard to the Ser-25 and Ser-296 sites, replacement of both residues caused a similar modest decrease in binding as was seen with dual substitution within the serine 18–20 site, while replacement of either Ser-25 or Ser-296 along with all three of the Ser-18, Ser-19, and Ser-20 residues was required to completely block the phosphorylation-induced increase in binding. These results show that each of the five serines identified by mass spectrometry can contribute to the phosphorylation-induced increase in PhLP binding to CCT and that no other CK2 phosphorylation sites are involved in this process, suggesting that all the major CK2 phosphorylation sites were identified in the mass spectrometric analysis.

It is important to note that the serine to alanine replacements did not change the binding of unphosphorylated PhLP to CCT significantly (Fig. 3A), indicating that the alanine substitutions did not affect the folding of the PhLP variants nor did they modify the PhLP-CCT interaction significantly. Thus, the loss of the phosphorylation-induced increase in binding with the multiple alanine substitutions could be attributed to an inability of the variants to be phosphorylated by CK2.

**Identification of Specific CK2 Phosphorylation Sites in Cells**—To assess whether the CK2 phosphorylation sites of PhLP identified in vitro were also phosphorylated in vivo, the decrease in electrophoretic mobility upon CK2 phosphorylation was exploited to detect PhLP phosphorylation events in living cells. In this experiment, PhLP serine to alanine substitution variants with a C-terminal myc tag were transfected into CHO cells. Cells were extracted, and the PhLP was immunoprecipitated and immunoblotted with an antibody to the tag to distinguish the variants from the endogenous PhLP. Substitution of a serine that normally would be phosphorylated by CK2 in wild-type PhLP would be expected to result in slower mobility of the CK2-phosphorylated PhLP variants.

![FIGURE 2. Mass spectrometric analysis of the CK2 phosphorylation sites of PhLP.](image-url)

A, PhLP was phosphorylated by CK2 in vitro and digested with trypsin, and the resulting peptide fragments were analyzed by LC/MS/MS. CID spectrum of the +2 parent ion corresponding to the m/z of the C-terminal sequence of PhLP plus one phosphate is indicated above the spectrum. The PhLP sequence ends at Asp-301, and the additional residues (smaller font) are part of the linker for the C-terminal myc tag of the recombinant human PhLP. The m/z values corresponding to the b and y ions resulting from fragmentation of this peptide are indicated. The 1764 m/z peak corresponds to the y16 ion with one dehydroalanine generated from the loss of H3PO4 from a phosphoserine during fragmentation. B, CID spectrum of the +2 parent ion corresponding to the m/z of residues 13–32 of PhLP plus one phosphate. The m/z values corresponding to the b and y ions resulting from fragmentation of this peptide are indicated. C, CID spectrum of the +2 parent ion corresponding to the m/z of residues 13–32 of PhLP plus two phosphates. The m/z values corresponding to the b and y ions resulting from fragmentation of this peptide are indicated.
in an increase in mobility of that PhLP variant. Wild-type PhLP showed decreased mobility of the entire band when compared with an unphosphorylated PhLP standard, indicating that all of the transfected PhLP was phosphorylated in the CHO cells (Fig. 3C, upper panel). The PhLP S20A variant showed two bands, a higher band with the same mobility as wild-type PhLP and a lower band

**FIGURE 3. Contribution of specific CK2 phosphorylation sites to the PhLP-CCT interaction.** A, phosphorylation-induced increase in PhLP binding to CCT is shown for several PhLP variants with the serine-to-alanine substitutions indicated. Binding of CCT to PhLP was determined for each variant as in Fig. 1. Representative immunoblots for the phosphorylated (P) and unphosphorylated (NP) variants are shown. B, the -fold increase in CCT bound upon CK2 phosphorylation of PhLP was calculated by dividing the CCT band intensity of the phosphorylated sample by that of the unphosphorylated sample. The graph gives the average increase ± S.E. from three to five separate experiments. C, the shift in electrophoretic mobility of PhLP upon CK2 phosphorylation was used to determine which of the putative CK2 sites were phosphorylated in cells. CHO cells were transfected with the indicated PhLP variants with C-terminal myc epitope tags. After 48 h, the cells were harvested and extracts were immunoprecipitated and immunoblotted with an antibody to the myc tag. Phosphorylation of the variants was determined by the shift in mobility of the PhLP band compared with wild-type PhLP-myc or purified, unphosphorylated PhLP-myc. D, the electrophoretic mobility of wild-type PhLP and the indicated PhLP variants after CK2 phosphorylation in vitro was also determined to compare the mobility shifts in vitro with those observed in cells. PhLP variants were analyzed by SDS-PAGE gels as in Fig. 1.
with increased mobility. The ratio of the intensities of the two bands was ~2 to 1, with the higher band having the greater intensity. The PhLP S18A and S19A showed a very small amount of the lower band, whereas S25A and S296A showed no lower bands. The presence of both bands in the S20A variant suggests that phosphorylation of Ser-18 or Ser-19 may be partially impaired when position 20 cannot be phosphorylated. These results indicate that Ser-20 is phosphorylated in cells and that other phosphorylation events might also occur within the serine 18–20 sequence.

A similar analysis was done with double and triple serine to alanine substitutions (Fig. 3C, middle panel). The S18A/S19A/S20A variant showed a single lower band compared with wild-type PhLP but that was still higher than the unphosphorylated control. The S18A/S20A and S19A/S20A variants also showed a major lower band, with almost no higher band corresponding to wild-type PhLP. The S18A/S19A variant showed both the higher and lower bands, confirming phosphorylation at Ser-20 and indicating that it is sufficient for the mobility shift. The two bands also suggest that Ser-20 phosphorylation may be impaired in the absence of serine or phospho-serine at position 18 or 19. In the case of the S25A/S296A variant, there was a single higher band with the same mobility as wild-type PhLP, demonstrating that the large decrease in mobility is not a result of Ser-25 or Ser-296 phosphorylation, but rather it stems from at least one phosphorylation event in the Ser-18, Ser-19, and Ser-20 sequence.

The mobility of PhLP variants substituted at four and all five sites was also determined. The S18A/S19A/S20A/S25A variant causes an additional increase in the mobility of the PhLP band to the same mobility as unphosphorylated PhLP, whereas the S18A/S19A/S20A/S296A variant did not increase the mobility beyond that of serines 18–20. The variant in which all five sites were substituted has the same mobility as the S18A/S19A/S20A/S25A variant and unphosphorylated PhLP. These data show that Ser-25 is also phosphorylated in cells, at least in the absence of phosphorylation at serines 18–20, and that Ser-25 phosphorylation causes a small decrease in PhLP mobility. The lack of change in mobility with substitution of Ser-296 did not permit a conclusion to be made about the phosphorylation of this site in cells. Either phosphorylation at Ser-296 did not occur or it did not change the mobility of PhLP in SDS gels.

A very similar pattern of electrophoretic mobility shifts was observed with in vitro CK2 phosphorylation of the PhLP S/A variants (Fig. 3D). S18A/S19A/S20A showed increased mobility compared with wild-type PhLP, and the S18A/S19A/S20A/S25A/S296A variant had the same mobility as unphosphorylated PhLP. The similarities in mobility of the PhLP variants between the in vitro phosphorylation and that found in cells argue that CK2 is responsible for PhLP phosphorylation in vivo, in agreement with previous data indicating that CK2 was the physiologically relevant kinase (14). Importantly, in the absence of CK2 phosphorylation, the S/A variants all had the same mobility as unphosphorylated wild-type PhLP (data not shown), indicating that the differences in mobility were not caused by the alanine substitutions. Together, these data make a strong case for CK2 phosphorylation events within the serines 18–20 and 25 sites in vivo.

**Mechanism of G Protein βγ Dimer Assembly**

**Effects of Specific CK2 Phosphorylation Sites on Gβγ Expression and Dimer Assembly**—It has recently been reported that substitution of all three serine residues in the serine 18–20 sequence blocked the ability of PhLP to enhance the cellular expression of Gβγ (24, 25). To further investigate this phenomenon, the CK2 phosphorylation site variants of PhLP were co-expressed with Gβ1 and Gγ2 in HEK-293 cells, and the effects on Gβγ expression were measured by immunoprecipitating the Gγ2 from cell extracts and immunoblotting for both Gβ1 and Gγ2. Co-expression of the single phosphorylation site variants did not change Gβγ expression significantly compared with wild type, nor did co-expression of the S18A/S19A or the S25A/S296A double variants (Fig. 4, A and B). However, co-expression of the S18A/S20A or the S19A/S20A double variants inhibited Gβ expression by 60–70% and Gγ expression by ~50% compared with wild type (Fig. 4A). Likewise, the S18A/S19A/S20A triple variant inhibited Gβ and Gγ expression by 70–80% and 60–70%, respectively (Fig. 4, A and B). Further substitution of Ser-25 and Ser-296 caused no further decreases in Gβγ expression (Fig. 4B). These data clearly show that phosphorylation of at least one serine within the serine 18–20 sequence is important for PhLP to assist in the expression of Gβγ, with Ser-20 phosphorylation contributing the most to this process. They also show that phosphorylation of Ser-25 and Ser-296 plays no additional role in Gβγ expression. Moreover, the significant reduction in Gβγ expression by several of the PhLP serine 18–20 variants to levels below those observed with the empty vector indicate that these variants block the ability of endogenous PhLP to support Gβγ expression and are thus acting as dominant negative inhibitors of Gβγ.

The reason for enhanced Gβγ expression in the presence of CK2-phosphorylated PhLP is that phosphorylated PhLP increases the rate of Gβγ dimer assembly (24). To determine which phosphorylation sites are critical for PhLP-mediated Gβγ assembly, the ability of the PhLP CK2 phosphorylation site variants to catalyze Gβγ dimer assembly was determined. All of the double and triple variants of the serine 18–20 sequence were compromised in their ability to assist in Gβγ dimer formation compared with wild-type PhLP (Fig. 4C). The S18A/S19A variant was the least compromised, as reflected by an assembly half-life of 99 min compared with 42 min for wild-type PhLP, whereas the S18A/S19A/S20A variant was the most compromised, with a half-life of 284 min (Fig. 4D). These data clearly show that phosphorylation at Ser-18 or 19 may be partially impaired

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FIGURE 4. Effects of PhLP phosphorylation on Gβγ expression and assembly. A, cellular expression of Gβγ dimers was determined in the presence of PhLP S18A/S19A/S20A variants. HEK-293 cells were transfected with Gβγ, HA-tagged Gγy, and the indicated variants. The cells were harvested, and extracts were immunoprecipitated with an antibody to the HA tag. The amount of HA-Gγy and co-immunoprecipitated Gβγ was determined by immunoblotting with anti-HA and anti-Gβ antibodies. A representative blot is shown. The graph gives the average Gβγ and HA-Gγy amounts ± S.E. relative to wild-type PhLP from three separate experiments. Cells in the empty sample were transfected with pcDNA3.1 vector with no PhLP cDNA. B, similar experiments were performed with PhLP variants S25A and S296A separately and in combination with S18A/S19A/S20A. The data are also combined from three separate experiments. C, the rate of nascent Gβγ dimer formation in the presence of CK2 phosphorylation site variants of PhLP was determined using a radiolabel pulse-chase assay. Time measurements indicate the sum of the 10-min pulse and the variable chase periods. A representative gel is shown. Band intensities were quantified, and molar ratios of Gβγ to HA-Gγy were calculated and plotted. Lines represent a fit of the data from three separate experiments to a first-order rate equation. Values for t½ are shown next to the graph.
observed with the other Ser-18, Ser-19, and Ser-20 variants is not. Together, the Gβγ assembly and expression data indicate that two phosphorylation events in the serine 18–20 sequence are required for PhLP to be fully active in catalyzing Gβγ assembly. Phosphorylation at one of the three sites results in partial activity, with Ser-20 phosphorylation conferring the most activity. The results also show that phosphorylation of Ser-25 or Ser-296 has no bearing on Gβγ assembly.

**Gβ γ Assembly and Interaction with CCT**—The correlation between the increase in binding of PhLP to CCT upon phosphorylation of serines 18–20 (Fig. 3B) and the necessity of phosphorylation of serines 18–20 for full activity in Gβγ assembly (Fig. 4C) suggests that the effects of PhLP phosphorylation on assembly may occur through CCT. However, a role for CCT in Gβγ assembly has not been established (24). If CCT does participate in the assembly process, then it must interact with Gβ, Gγ, or both. An interaction between Gβ and CCT has been observed in yeast protein interaction screens, but no such interaction has been reported in mammalian cells. Therefore, the binding of Gβ and Gγ to CCT was assessed by co-immunoprecipitation of overexpressed Gβ or Gγ in HEK-293 cells. Gβ co-immunoprecipitated with CCT robustly, to a similar extent as overexpressed PhLP, whereas overexpressed Pdc, which does not bind CCT, was not found in the CCT immunoprecipitate (Fig. 5A). Thus, Gβ appears to be specifically interacting with CCT under overexpression conditions. In contrast, overexpressed Gγ did not co-immunoprecipitate with CCT (Fig. 5A). To determine whether the interaction also occurred with endogenous amounts of Gβ, the experiment was also done without overexpressing Gβ. Co-immunoprecipitation of Gβ with CCT was also observed with endogenous Gβ, confirming the results of the overexpression experiments (Fig. 5B).

The manner in which PhLP binds CCT at the top of the apical domains without entering the folding cavity (18) suggests that PhLP, Gβ, and CCT might form a ternary complex in the process of Gβγ folding. If such a ternary complex does exist, then PhLP would be predicted to increase the binding of Gβ to CCT and vice versa. To test this possibility, the effects of PhLP or Gβ overexpression on the binding of the other to CCT was measured. As predicted, Gβ overexpression increased the binding of endogenous PhLP to CCT (Fig. 5C). However, PhLP overexpression unexpectedly caused a small but reproducible decrease in Gβ binding to CCT (Fig. 5B). It is possible that this decrease in Gβ binding to CCT might be caused by PhLP-catalyzed Gβγ assembly and release of the Gβγ dimer from CCT. To test this possibility, the effects of two PhLP variants that do not support Gβγ assembly on Gβ binding to CCT were also tested. One variant was PhLP S18A/S19A/S20A, and the other was a truncation variant in which residues 1–75 had been removed (PhLP Δ1–75) (24). Both of these variants bind CCT, but they block Gβγ assembly in a dominant negative manner (24). Overexpression of either of these variants increased endogenous Gβ binding to CCT dramatically (Fig. 5B). Thus, it appears that in the absence of serine 18–20 phosphorylation, PhLP forms a ternary complex with Gβ and CCT that cannot progress in the assembly process. It is interesting to note that the PhLP Δ1–75 variant binds Gβγ very poorly (24), yet it is still able to stabilize the complex between Gβ and CCT. This observation indicates that PhLP Δ1–75 may do so, more through interactions with CCT than through interactions with Gβ.

**PhLP Phosphorylation Is Required for the Release of Gβ from CCT and Interaction with Gγ**—To further investigate the apparent correlation between the destabilization of the PhLP/Gβ-CCT ternary complex by PhLP phosphorylation and the requirement for PhLP phosphorylation in Gβγ assembly, the effects of Gγ on ternary complex formation with several PhLP variants was measured. Gβ was overexpressed in HEK-293 cells with Gγ and PhLP variants as indicated, and the amount of Gβ co-immunoprecipitating with CCT was measured (Fig. 6A). Co-expression of Gγ caused a decrease in Gβ binding to CCT that was intensified by the co-expression of
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A

B

C

D

| Transfection conditions | Gβ dissociation rate (t_{1/2}, h) |
|-------------------------|----------------------------------|
| Gβ                      | 7.5 ± 2.5                        |
| Gβ, PhLP S18-20A        | 8.8 ± 2.3                        |
| Gβ, PhLP WT             | 2.2 ± 0.4                        |

| Transfection conditions | Gβ dissociation rate (t_{1/2}, h) |
|-------------------------|----------------------------------|
| Gβ, Gγ                  | 3.3 ± 0.5                        |
| Gβ, Gγ, PhLP S18-20A    | 41 ± 31                          |
| Gβ, Gγ, PhLP WT         | 2.3 ± 0.5                        |

CCT IP

Lysate
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FIGURE 6. PhLP phosphorylation is required for the release of Gβ from CCT and interaction with Gγ. A, the effects of PhLP phosphorylation and Gγ co-expression on Gβ binding to CCT were measured by co-immunoprecipitation. HEK-293 cells were transfected with FLAG-Gβ, HA-Gγ, and the PhLP variants as indicated. Cells extracts were immunoprecipitated with an antibody to CCT and then immunoblotted for FLAG-Gβ. A representative immunoblot is shown. Bars in the graph represent the average ± S.E. of the Gβ band intensity relative to the Gβ/PhLP-WT sample from three separate experiments. B, the effects of PhLP phosphorylation on the rate of Gβ release from CCT were measured using a radiolabel pulse-chase assay. HEK-293 cells were transfected with FLAG-Gβ, and the indicated PhLP variants. The pulse-chase assay was performed as in Fig. 4C. After the chase times indicated, cell extracts were immunoprecipitated with antibodies to CCT or Gβ. Proteins were resolved by SDS-PAGE, and radiolabeled bands were detected using a PhosphorImager. The Gβ band intensities were quantified, and ratios of nascent Gβ from CCT upon PhLP overexpression in the absence of Gγ overexpression (Fig. 6B) is consistent with the second mechanism in which a phosphorylated PhLP-Gβ complex would be released prior to Gγ binding to Gβ. This result would not be expected in the first mechanism in which Gγ binding would be required for release of Gβ from CCT. Similarly, the observed lack of increase in the Gβ dissociation rate upon co-expression of Gγ with PhLP would be predicted by the second mechanism but not by the first. On the other hand, the increased release of Gβ from CCT upon Gγ overexpression in the absence of PhLP overexpression is consistent with the first mechanism, but this result could also be explained by the second mechanism if the endogenous PhLP were acting catalytically to release Gβ from CCT for association with Gγ. In this case, the dissociation process would be drawn forward by the formation of the Gβγ dimer and its association with Ga and cell membranes (Fig. 7).

To further assess the role of Gγ in the release of Gβ from CCT, the possible association of Gγ with Gβ and PhLP in CCT complexes was determined. Gγ was co-expressed with the indicated combinations of Gβ and the PhLP variants, the CCT complexes were immunoprecipitated, and the samples were immunoblotted for Gγ. Gγ was not found in any of the CCT immunoprecipitates (Fig. 6D), despite the fact that Gβ and PhLP could be readily found under these conditions (see Fig. 5). Thus, it appears that Gγ does not interact with CCT in any of its complexes with Gβ and PhLP.

Together, the data in Fig. 6 indicate that PhLP phosphorylation results in the release of a PhLP-Gβ complex from CCT that can then associate with Gγ to form the Gβγ dimer. This conclusion is also supported by the previously reported observation that PhLP forms a stable complex with Gβ that does not include Gγ (24).

DISCUSSION

A model for Gβγ Assembly—Recent studies have shown that PhLP acts as an essential chaperone in the assembly of Gβγ dimers by binding the Gβ subunit and thereby allowing Gγ to associate with Gβ (24, 25). Phosphorylation of PhLP at serines 18–20 by CK2 was required for Gβγ assembly to

wild-type PhLP. In striking contrast, Gβ binding to CCT was greatly enhanced by co-expression of PhLP Δ1–75 and was completely insensitive to co-expression of Gγ. Co-expression of PhLP S18A/S19A/S20A also enhanced Gβ binding to CCT significantly, and Gγ had much less of an effect on binding than with wild-type PhLP. Interestingly, the effects of PhLP Δ1–75 and S18A/S19A/S20A on Gβ binding to CCT in the presence of Gγ were quantitatively very similar to their effects on Gβγ assembly. PhLP Δ1–75 completely blocked Gβγ assembly (24) and Gγ-mediated dissociation of Gβ from CCT, whereas PhLP S18A/S19A/S20A decreased the rate of Gβγ assembly by 15-fold (24) and Gγ-induced dissociation of Gβ from CCT by 9-fold (compare the Gβγ PhLP-WT sample to the Gβγ PhLP S18A/S19A/S20A sample in Fig. 6A). From these data, it appears that PhLP phosphorylation contributes to Gβγ assembly by enhancing the ability of Gγ to release Gβ from the ternary complex.

There are two possible mechanisms by which phosphorylated PhLP could contribute to Gγ-mediated release of Gβ from CCT. Both involve a conformational change in the ternary complex upon PhLP phosphorylation. First, PhLP phosphorylation could induce a conformation that allows Gγ to access Gβ in the ternary complex and form the Gβγ dimer. The Gβγ would then be released from CCT. Second, phosphorylation could induce a conformation that releases PhLP-Gβ from CCT, thereby freeing the Gγ binding site on Gβ for Gβγ association to occur. To distinguish between these two mechanisms, the effects of Gγ and PhLP overexpression on the rate of dissociation of Gβ from CCT were measured in this experiment. In cells co-expressing Gβ with Gγ, PhLP, or PhLP S18A/S19A/S20A were pulsed with [35S]methionine for 10 min to label the nascent polypeptides and then were chased with unlabeled methionine. At the times indicated, the cells were lysed and CCT was immunoprecipitated. The co-immunoprecipitating proteins were separated by SDS-PAGE, and the amount of [35S]S in the Gβ band was quantified. In the absence of PhLP or Gγ co-expression, the dissociation rate of nascent Gβ from CCT was very slow, with a t1/2 of ~8 h. PhLP co-expression increased the rate by 4-fold to a t1/2 of ~2 h. In contrast, PhLP S18A/S19A/S20A co-expression did not increase the dissociation rate (Fig. 6B). When Gγ was co-expressed with Gβ, the dissociation rate increased by ~2-fold to a t1/2 of ~3 h, whereas, when both Gγ and PhLP were co-expressed, the t1/2 increased even further to ~2 h, the same value observed in the absence of Gγ overexpression (Fig. 6C). When PhLP S18A/S19A/S20A was co-expressed with Gγ, there was essentially no Gβ dissociation, similar to what was seen in the absence of Gγ overexpression (Fig. 6C). These effects of Gγ, PhLP, and PhLP S18A/S19A/S20A on the dissociation rates are consistent with their effect on the steady-state binding of Gβ to CCT (Fig. 6A) and further demonstrate that PhLP phosphorylation is required for the release of Gβ from the ternary complex. These findings are able to distinguish between the two potential mechanisms mentioned above. For example, the enhanced rate of dissociation of Gβ from CCT upon PhLP overexpression in the absence of Gγ overexpression (Fig. 6B) is consistent with the second mechanism in which a phosphorylated PhLP-Gβ complex would be released prior to Gγ binding to Gβ. This result would not be expected in the first mechanism in which Gγ binding would be required for release of Gβ from CCT. Similarly, the observed lack of increase in the Gβ dissociation rate upon co-expression of Gγ with PhLP would be predicted by the second mechanism but not by the first. On the other hand, the increased release of Gβ from CCT upon Gγ overexpression in the absence of PhLP overexpression is consistent with the first mechanism, but this result could also be explained by the second mechanism if the endogenous PhLP were acting catalytically to release Gβ from CCT for association with Gγ. In this case, the dissociation process would be drawn forward by the formation of the Gβγ dimer and its association with Ga and cell membranes (Fig. 7).

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A model for Gβγ Assembly—Recent studies have shown that PhLP acts as an essential chaperone in the assembly of Gβγ dimers by binding the Gβ subunit and thereby allowing Gγ to associate with Gβ (24, 25). Phosphorylation of PhLP at serines 18–20 by CK2 was required for Gβγ assembly to
occur, yet the means by which phosphorylation of serines 18–20 contributes to assembly was unknown. Moreover, CCT had been implicated in the assembly process, but the results were conflicting (18, 24, 25). The current study provides evidence for a molecular mechanism describing both the role of CCT and PhLP phosphorylation in G\(\beta\)H9252/H9253 assembly (Fig. 7). There are five important steps in this mechanism: 1) the nascent G\(\beta\) polypeptide binds CCT. This is a stable complex that releases G\(\beta\) very slowly in the absence of PhLP. 2) PhLP binds forming a ternary complex. If PhLP is not phosphorylated, then the ternary complex assembles in a conformation that readily releases the PhLP/G\(\beta\) dimer. 3) PhLP-G\(\beta\) dissociates from CCT. The structure of the Pdc-G\(\beta\) complex shows that Pdc binds G\(\beta\) on the opposite face as G\(\gamma\) (29), predicting that the G\(\gamma\) binding site on G\(\beta\) would be free in the PhLP-G\(\beta\) dimer. 4) G\(\gamma\) binds G\(\beta\) forming a PhLP-G\(\beta\)\(\gamma\) complex. This complex is stable with a 100 nM binding affinity (11). However, both the G\(\alpha\) binding site and the membrane association surface of G\(\beta\)\(\gamma\) overlap extensively with the PhLP binding site (11); therefore in the presence of G\(\alpha\) and membranes, PhLP would be expected to be released from G\(\beta\)\(\gamma\). 5) G\(\beta\)\(\gamma\) associates with G\(\alpha\) and/or membranes in a manner yet to be defined. In the process, PhLP is released to catalyze another round of dimer formation. The approximate position of the serine 18–20 phosphorylation site is depicted by a red oval marked ‘P’. The relative amount of positive and negative charges on the CCT apical domains that contact the PhLP N-terminal domain is also indicated. See text for details.
some cell types, whereas in others assembly is highly regulated, only occurring under certain conditions that promote CK2 phosphorylation of PhLP.

These investigations into the mechanism of PhLP-mediated Gβγ dimer assembly and its regulation by CK2 phosphorylation suggest that PhLP and its interactions with Gβγ and CCT could be targeted by therapeutics to control the levels of Gβγ expression and thus the degree of G protein signaling within the cell, perhaps providing additional tools to treat the myriad of G protein-linked diseases.

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would form a PhLP-Gβ complex that would not accept Gγ (24), are incomplete.

Phosphorylation-induced Conformational Changes—One apparent inconsistency in the data is that CK2 phosphorylation of PhLP increased its binding to CCT in the absence of Gβ (Fig. 1), yet PhLP phosphorylation was necessary for the release of PhLP-Gβ from CCT in the presence of Gβ (Figs. 4–6). The difference between these observations may stem from differences in the structures of the PhLP-CCT and PhLP-Gβ-CCT complexes. Clues regarding the nature of the phosphorylation-dependent changes in these structures may be gleaned from the cryoelectron microscopic studies of the unphosphorylated PhLP-CCT complex (18). In this complex, PhLP was shown to interact in two distinct conformations at the top of the CCT toroid, contacting only the CCT apical domains (18). In one interaction, the phosphorylation site of PhLP was in close proximity to the CCTα and -ε apical domains and in the other conformation, the phosphorylation site was in close proximity to the CCTζ and -β apical domains. The binding surfaces of all eight apical domains are dominated by charged surfaces of all eight apical domains are dominated by charged and polar residues (31) with the CCTα and -β binding surfaces having a high distribution of negative charge, whereas the CCTζ binding surface exhibits an extensive positively charged patch. The serine 18–20 phosphorylation site of PhLP is harbored within a sequence 18SSDEDES26 that is already very negatively charged. The addition of phosphates within this sequence would create an extremely high concentration of negative charge that would interact effectively with the positively charged patch of CCTζ. In the absence of Gβ, phosphorylation could favor the conformation that brings the PhLP phosphorylation site in close proximity to the CCTζ apical domain, increasing the binding of PhLP to CCT. In the presence of Gβ, it is possible that interactions with Gβ may limit the ability of PhLP to rotate on the top of the CCT toroid. Thus, the phosphorylation site may be fixed in close proximity to the CCTα and -ε apical domains, causing electrostatic repulsion between the negative charges on the CCTα and -ε binding surfaces and the PhLP phosphorylation site. This repulsion might destabilize the ternary complex and allow the release of the PhLP-Gβ complex. Further studies will be required to test the validity of this structural model.

Regulation of CK2 Phosphorylation of PhLP—Given the essential role of CK2 phosphorylation of PhLP in Gβγ dimer formation, an important issue yet to be addressed is the regulation of this phosphorylation event. CK2 is a constitutively active kinase with many protein substrates (32). Determination of which substrates are phosphorylated and when phosphorylation occurs appears to be controlled by regulated expression and assembly of the CK2 α,β,γ tetramer and by the association of different CK2 binding partners (32). In the case of PhLP, CK2 phosphorylation occurs within the first 30 min of its synthesis (data not shown), and it remains completely phosphorylated under the cell culture conditions used here (Fig. 2). It is not clear from the current data whether phosphorylation occurs prior to or after association of PhLP with CCT (Fig. 7). In mouse tissues, PhLP was also completely phosphorylated in brain and heart but was mostly unphosphorylated in the adrenal gland (14). It is possible that Gβγ assembly is a continuous process in
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