The Mechanism of Assembly of the G-Protein Beta Gamma Subunit Dimer by CK2 Phosphorylated Phosducin-Like Protein and the Chaperonin Containing TCP-1

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by

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Phosducin-like protein (PhLP) binds G-protein βγ subunits and is thought to assist in assembly of the Gβγ dimer. Phosphorylation of PhLP at serine residues 18-20 by the casein kinase 2 (CK2) appears to play an essential role in this process. PhLP has also been shown to interact with the chaperonin containing TCP-1 (CCT) atop its apical domain, not entering the substrate folding cavity. However, the physiological role of the PhLP-CCT interaction in Gβγ dimer formation remains unclear. This study addresses the mechanism of Gβγ assembly by exploring the specific roles of CCT and CK2 phosphorylation of PhLP in the assembly process. Both overexpressed and endogenous Gβ were shown to co-immunoprecipitate with CCT to a similar extent as PhLP, indicating that CCT may be involved in the folding of Gβ. In addition, Gβ overexpression enhanced the binding of PhLP to CCT, suggesting the formation of a ternary PhLP-Gβ-CCT complex. In contrast, overexpression of PhLP caused the release of Gβ from CCT. This release was blocked by a PhLP S18-20A variant that lacks the S18-20 CK2 phosphorylation site. PhLP S18-20A has been previously shown to negatively affect Gβγ dimer formation, suggesting a correlation between PhLP-mediated release of Gβ from CCT and Gβγ assembly. Experiments investigating the role of Gγ in this process show that Gγ does not interact with CCT nor is it the essential factor in the release of Gβ from CCT. A new model is therefore proposed for Gβγ assembly
involving the formation of a PhLP-Gβ-CCT ternary complex followed by the release of a phosphorylated PhLP-Gβ complex from CCT. In the PhLP-Gβ complex, the Gγ binding face of Gβ is exposed, allowing for the formation of the Gβγ dimer.
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ABBREVIATIONS

AC  Adenylyl cyclase
CCT  Chaperonin Containing Tailless complex polypeptide 1 (TCP-1)
CHO cells  Chinese hamster ovary cells
CK2  Casein kinase 2
Cryo-EM  Cryoelectron microscopy
Gα  G-protein alpha subunit
Gβ  G-protein beta subunit
Gβγ  G-protein beta and gamma subunit dimer
Gγ  G-protein gamma subunit
GPCR  G-protein coupled receptor
HEK-293 cells  Human embryonic kidney -293 cells
Pdc  Phosducin
PhLP  Phosducin-like protein
RGS  Regulators of G protein signaling
WT  Wild-type
CHAPTER 1
INTRODUCTION TO G-PROTEIN SIGNALING, PHOSDUCIN-LIKE PROTEINS, AND THE CYTOSOLIC CHAPERONIN CONTAINING TCP-1

In order for cells to respond to their environment, extracellular stimuli must be able to elicit an intracellular response. Cellular receptors are one method of facilitating this process. The superfamily of G-protein coupled receptors (GPCRs) comprises roughly two percent of human genes, contributing substantially to cell signaling and communication (Ben-Shlomo et al., 2003). Because of their significance and wide range of functionality, drug development often targets GPCRs and the G-protein pathway, making it essential to better understand their modes of action.

Although most GPCRs display a high degree of ligand specificity, the fact that there are a large number of GPCRs within the superfamily permits a wide variety of ligands to stimulate a G-protein-mediated response. GPCRs contain seven transmembrane helices whose hydrophobicity allows them to span the plasma membrane (Ben-Shlomo et al., 2003; Preininger and Hamm, 2004; Sealfon, 2005, Wettschureck and Offermanns, 2005). Ligand binding and transmembrane regions vary between families, giving the receptors specificity. However, conserved essential residues among GPCRs allow them to function similarly in activation of G-proteins (Palczewski et al., 2000).

A model for GPCRs comes from rhodopsin, the abundant, well-characterized, retinal GPCR (Fig. 1.1). Upon activation, rhodopsin undergoes a conformational change, moving helix VI in relation to helix III and altering the intracellular face of the GPCR.
This movement enhances G-protein association with the GPCR resulting in activation of the G protein (Farrens et al., 1996).

**Figure 1-1. GPCR structure and mechanism of activation.** Rhodopsin is used as a model for the structure and mechanism of activation of GPCRs. (A) A 2-dimensional representation of rhodopsin shows the seven helices of the GPCR spanning the plasma membrane. The N-terminus is located extracellularly and the C-terminus is found intracellularly. (B) A ribbon diagram models the packing of the GPCR helices (Palczewski et al., 2004). (C) Rotation of helix VI (f) in relation to helix III (c) causes a conformational change that enhances G-protein binding to the intracellular side of the GPCR (Dunham and Farrens, 1999).
G-proteins are heterotrimers made up of α, β, and γ subunits that associate with the intracellular surface of the plasma membrane. Binding to an activated receptor causes the dissociation of GDP from Gα, allowing GTP to enter in its place in the nucleotide binding site. GTP binding causes a conformational change in the Gα subunit, destabilizing its interaction with Gβγ, resulting in the dissociation of Gα-GTP from Gβγ. Each complex is then free to interact with its respective effectors. Activation of G-proteins allows them to target a variety of effectors, thereby propagating the original extracellular signal (Cabrera-Vera et al., 2003; Wettschureck and Offermanns, 2005).

The G protein is turned off by the hydrolysis of GTP to GDP on the Gα subunit, making it available to reassociate with Gβγ and return to the plasma membrane to await activation once again.

Although Gα contains some intrinsic GTPase activity, the rate at which it hydrolyzes GTP in vitro is much slower than under physiological conditions, suggesting that other proteins are involved in accelerating this process in vivo (Cabrera-Vera et al., 2003; Wettschureck and Offermanns, 2005). One family of proteins, regulators of G-protein signaling (RGS), were discovered and named accordingly because of their role in assisting Gα’s hydrolysis of GTP to GDP. RGS proteins act by stabilizing Gα in its GTPase active conformation. Although they do not directly contribute any catalytic residues, RGS proteins stabilize the position of a key glutamine residue of Gα which orients a water molecule required for nucleophilic attack in GTP hydrolysis (Tesmer et al., 1997). Regulation of RGS proteins and the rate of subsequent GTP hydrolysis determines the duration of the G protein’s affect on its effectors.
The 16 human genes encoding Gα subunits can be grouped into four main families—Gαs, Gαq/11, Gα12/13, and Gαi/o, with each family containing several genes that target similar effectors. Gαs primarily activates isoforms of adenylyl cyclase (AC) while Gαq/11 stimulates isoforms of phospholipase C-β (PLC-β). Gα12/13 has a large number of effectors varying from Na+/H+ exchangers to RhoA GTPase activating proteins. Gαi/o family members also have a variety of effectors with inhibition of AC and activation of cGMP phosphodiesterase as the principle targets. Because Gαi/o is often found in higher concentrations, its dissociation from its Gβγ subunits is thought to allow Gβγ to activate its downstream effectors (Wettschureck and Offermanns, 2005).

Originally, Gβγ was thought to lack its own effectors and only facilitate G-protein signaling by assisting Gα’s interaction with the plasma membrane and receptors. However, the existence of five different Gβ subunits and twelve Gγ subunits in the human genome suggest this first hypothesis is unlikely (Cabrera-Vera et al., 2005; Wettschureck and Offermanns, 2005). Four of the five Gβ subunits bind Gγ tightly and these Gβγ complexes have been shown more recently to have a number of effectors. Note that Gβ5 has less sequence homology than the other four Gβ subunits and binds Gγ weakly. Although Gβ5 has some in vitro interactions with the Gγ2, it displays greater affinity for the Gγ-like domain of RGS proteins, suggesting a slightly different contribution to G-protein signaling (Yoshikawa et al., 2000; Wettschureck and Offermanns, 2005). The other Gβγ dimers are known to upregulate isoforms of PLC-β and the β-adrenergic receptor kinase and to downregulate isoforms of AC (Cabrera-Vera et al., 2003; Wettschureck and Offermanns, 2005). Gβγ also regulates ion-channels,
activating G-protein-activated inwardly rectifying K\(^+\) channels (GIRKs) and inhibiting voltage-gated Ca\(^{2+}\) channels (Dascal, 2001). As the number of known G\(\beta\gamma\) effectors continues to grow, it becomes increasingly important to understand the regulation of these subunits.

Two G\(\beta\gamma\) binding partners have been described as regulators of G\(\beta\gamma\) signaling: phosducin (Pdc) and phosducin-like protein (PhLP). While Pdc is primarily found in the retina and pineal gland, PhLP is widely expressed in a variety of tissues. Both Pdc and PhLP are members of the Pdc gene family, which consists of three subgroups: Pdc-I, Pdc-II, and Pdc-III. Both Pdc and PhLP are members of the Pdc-I subgroup (Blaauw et al., 2003), and they both bind G\(\beta\gamma\) with high affinity (Savage et al., 2000). PhLP isoforms in subgroups II and III do not bind G\(\beta\gamma\), and all future references to PhLP will refer to the member of the Pdc-I family.

PhLP shares a 41% amino acid identity and a 65% homology with Pdc (Miles et al. 1993). Both Pdc and PhLP’s interactions with the G\(\beta\gamma\) were initially thought to be inhibitory in nature, preventing G\(\beta\gamma\) from performing its normal functions (Bauer et al., 1992; Lee et al., 1992; Schroder and Lohse, 1996; McLaughlin et al., 2002a). However, disruption of the PhLP gene in the chestnut blight fungus *Cryphonectria parasitica* (Kasahara et al., 2000), the soil amoeba *Dictyostelium discoideum* (Blaauw et al., 2003), and most recently the fungus *Aspergillus nidulans* (Seo and Yu, 2006) and the nematode *Caenorhabditis elegans* (Lacosta et al., 2006) show physiological phenotypes very similar to those of G\(\beta\) null mutants. These findings indicate that PhLP may act as a positive rather than a negative regulator of G-protein signaling.
Another insight into the role of PhLP comes from proteomic analyses to identify its binding partners. These analyses identified the chaperonin containing TCP-1 (CCT) as a major PhLP binding partner (McLaughlin et al. 2002b). CCT is a large protein complex that assists in the folding of nascent polypeptides into their native structures (Valpuesta et al., 2002). It is similar in many respects to the prokaryotic chaperone GroEL. CCT is comprised of two rings placed back to back to form a toroid structure. Each ring contains eight different subunits that are conserved between the two rings. ATP binding on one side of CCT causes a conformational change allowing for protein folding in the other side (Llorca et al., 2001). Unlike GroEL, CCT displays substrate specificity. Actin and tubulin are two of the well known substrates of CCT, but the list continues to grow as more proteins are shown to require CCT to fold to their native conformations (Thulasiraman et al., 1999; Siegers et al., 2003). Despite its high affinity for the chaperonin, PhLP does not require CCT to fold into its native state. Denatured PhLP spontaneously refolds in the absence of CCT, and native PhLP exhibits a two-fold increase in binding affinity for CCT over that of denatured PhLP (McLaughlin et al. 2002b).

PhLP-CCT complexes visualized through cryo-electron microscopy (cryo-EM) indicate that PhLP does not bind in the folding cavity of CCT but rather sits atop CCT’s apical domains (Martin-Benito et al. 2004, Fig. 1.2). This interaction exhibits some similarities to the binding of the co-chaperone prefoldin to CCT, though PhLP only binds one of the two rings in a one-to-one PhLP:CCT stoichiometry, whereas prefoldin binds in a two-to-one prefoldin:CCT complex (Martin-Benito et al., 2002). There appear to be two major conformations for the PhLP-CCT interactions with PhLP binding subunits on
opposite sides of the ring. In 65% of the complexes, PhLP interacts with CCT subunits \( \gamma/\theta \) on one side and \( \alpha/\varepsilon/\zeta \) on the other side. The other 35% of the complexes consists of interactions with \( \delta/\eta \) on one side and \( \zeta/\beta/\gamma \) on the other (Martin-Benito et al. 2004). These apical interactions prevent other proteins from entering the folding site of CCT.

Overexpression of PhLP in CHO cells inhibits CCT-dependent proteins from binding and folding at normal levels, suggesting a role of PhLP as a regulator of protein folding (McLaughlin et al. 2002b, Martin-Benito et al. 2004). Combining these structural observations with the findings from the genetic studies indicating that PhLP is required for \( G\beta \) function leads to the hypothesis that PhLP and CCT may participate in \( G\beta\gamma \) folding and complex assembly.

**Figure 1-2. PhLP binds on top of the apical domain of CCT.** Computer modeling from CryoEM data shows PhLP interactions with CCT. (A) Top and (B) side views of show PhLP binding atop CCT and occluding CCT’s protein folding cavity. (C) CCT in its un-bound state. (D) and (E) show PhLP interactions with specific subunits of CCT. Conformation (D) was observed to occur 65% of the time, and conformation (E) the other 35% (Martin-Benito et al., 2004).
This hypothesis was tested in a recent study measuring the effects of siRNA-mediated inhibition of PhLP expression nascent Gβγ dimer assembly (Lukov et al., 2005). PhLP knockdown in mammalian cells decreased the total Gβ protein present in the cell without changing the Gβ mRNA level. This result suggested a translational or post-translational affect of PhLP on Gβ. Further investigation showed that the ability of Gβ to assemble into dimers with Gγ was impaired upon siRNA-mediated depletion of PhLP. The rate at which Gβγ assembly occurred was tightly linked to PhLP expression levels. A 4-fold reduction in PhLP resulted in a five-fold decrease in the rate of Gβγ assembly, while PhLP overexpression enhanced the rate of assembly by 4-fold (Lukov et al., 2005).

In attempting to understand what contributes to this phenomenon, PhLP variants with alanine substitutions at serine residues 18-20 were created. These serine residues have been shown to be actively phosphorylated by CK2 both \textit{in vitro} and \textit{in vivo} and appear to contribute to PhLP’s regulation of Gβγ (Humrich, et al., 2003; Lukov et al., 2006). Overexpression of PhLP mutant S18-20A, which cannot be phosphorylated at those residues, decreases Gβγ expression by 70-80%. Furthermore, PhLP S18-20A overexpression decreased the rate of Gβγ assembly by 4-fold below the control while endogenous PhLP overexpression increased the rate of assembly by 4-fold above the control, a disparity of almost 16-fold between PhLP-WT and PhLP S18-20A (Lukov et al., 2005). It appears then that CK2 phosphorylation of PhLP at residues 18-20 is necessary for PhLP-mediated assembly of Gβγ. Note that another study shows that CK2 phosphorylation at these residues appears to have no affect on PhLP binding to Gβγ, while causing a 7-fold increase in the binding of PhLP to CCT, suggesting that CCT may
also participate in this assembly process (Lukov et al., 2006). However, a PhLP variant which lacks the first 75 N-terminal residues (PhLP Δ1-75) binds Gβ with low affinity but binds CCT well. This variant was even more effective in inhibiting Gβγ expression and assembly than the PhLP S18-20A variant. Furthermore, another PhLP variant (PhLP 132-135A) that binds Gβ normally but CCT very poorly shows no negative effect on the rate of Gβγ assembly compared to PhLP-WT (Lukov et al., 2005).

Clearly the roles of PhLP-CCT and PhLP-Gβγ interactions in Gβγ assembly remain inconclusive, particularly in relationship to each other. From these studies it appears that the PhLP-CCT interaction is not necessary for Gβγ assembly while the PhLP-Gβγ interaction and CK2 phosphorylation of PhLP are essential. In contrast, another study shows that siRNA-mediated depletion of CCT leads to a decrease in Gβγ expression, a similar affect to that of the PhLP S18-20A and PhLP Δ1-75 variants (Humrich et al., 2005; Lukov et al., 2005). The following research reconciles this disparity and addresses the role of both PhLP and CCT in the Gβγ assembly process by showing the formation of a ternary PhLP-Gβ-CCT complex. Furthermore, the role of PhLP phosphorylation in this process is shown to mediate the release of Gβ from CCT thereby allowing it to interact with Gγ. A new model is therefore proposed for the assembly of Gβγ through the formation of a ternary PhLP-Gβ-CCT complex.
CHAPTER 2
EXPERIMENTAL PROCEDURES

Materials – DMEM F-12 50/50 was purchased from Mediatech, Inc. Lipofectamine PLUS reagent was from Invitrogen. The Coomassie Plus Protein Assay reagent was from Pierce. ECL Plus Western blotting chemiluminescence reagents and \(^{35}\text{S}\) Methionine were from Amersham Biosciences. The anti-G\(\beta_1\) antibody was prepared as described (Lukov et al., 2004) and then affinity purified on a peptide affinity column. The anti-PhLP antibody was also prepared as described previously (Thulin et al., 1999). Anti-CCT-\(\epsilon\) (Serotec), anti-c-myc (BioMol), anti-Flag (Sigma-Aldrich) and anti-HA (Roche) antibodies were purchased from the commercial sources indicated. Anti-mouse, anti-rat, and anti-rabbit horseradish peroxidase-conjugated secondary antibodies were purchased from Calbiochem. Human embryonic kidney (HEK)-293 cells were obtained from the American Type Cell Culture repository. Midi-prep kits used for DNA preparation, L-Glutamine, IGEPAL (NP-40), and protease inhibitor cocktail were all from Sigma Aldrich. PMSF was purchased from Spectrum Chemicals. Fetal bovine serum was from HyClone. Protein A/G beads used for immunoprecipitation were from Santa Cruz Biotechnology and PBS was from Fisher.

Cell Culture – HEK-293 cells were grown in DMEM F-12 50/50 supplemented with 10% fetal bovine serum. Cells were grown at 37°C with 5% \(\text{CO}_2\) and were subcultured regularly to maintain viability. Cells were not used beyond 25 passages.

cDNA constructs – Human PhLP and Pdc were cloned into the pcDNA 3.1 B vector containing c-myc and His\(_6\) tags (Invitrogen) as described (Carter et al., 2004;
Lukov et al., 2005). PhLP variants S18-20A and Δ1-75 were both created using PCR based methods as described (Lukov, 2005). N-terminal HA-tagged Gγ2 and N-terminal Flag-tagged Gβ1 cDNA in pcDNA 3.1 were obtained from the UMR cDNA Resource Center.

CCT Co-immunoprecipitation - HEK-293 cells were split and plated in 6-well plates, or 100 mm dishes for endogenous Gβ experiments, to be 70-80% confluent the following day. Cells were then transfected with the indicated cDNAs using Lipofectamine Plus reagent. After 40-48 hours, the cells were washed with PBS and solubilized in 200 µl of immunoprecipitation buffer (~2% IGEPAL (NP-40) in PBS) per well or 1.2 ml per dish. For each ml of this solution, 6 µl of 100 mM PMSF and 4.5 µl of Sigma protease inhibitor cocktail were added. The cells were passed 10 times through a 25 gauge needle and then centrifuged at 14,000 rpm for 10 minutes. If protein concentration determinations were necessary, 10 µl of sample was removed and diluted in 40 µl H2O and analyzed with the Coomassie Plus assay. If the protein concentration disparity was greater than 10%, samples were adjusted with immunoprecipitation buffer to equalize the protein concentrations.

CCT was immunoprecipitated by first adding 2.5 µg anti-CCTε per well, 3.5 µg per 2-wells for endogenous PhLP, or 10 µg per 100 mm dish for endogenous Gβ and incubated with rotation at 4°C for at least 30 minutes. This was followed by the addition of 25, 35, or 60 µl of a 50% slurry of protein A/G beads, depending on the amount of antibody added, followed by a second 30 min incubation. After three washes with immunoprecipitation buffer, complexes were solubilized in 4X SDS sample loading buffer. Samples were then boiled for 5 minutes, with the exception of endogenous Gβ
samples which were not boiled, and then resolved on 10% Tris-Glycine SDS-PAGE or 16.5% Tris-Tricine SDS-PAGE gels for Gγ detection.

Proteins were transferred to a nitrocellulose membrane and blots were blocked in 5% non-fat dry milk in TBS-T. Anti-HA Gγ blots were blocked in 2% BSA or 2% non-fat dry milk in TBS. Primary antibodies were diluted in blocking buffers and incubated with rotation overnight and secondary antibodies were diluted in TBS-T or TBS and incubated for 45-60 minutes with rocking. Between primary and secondary incubations and before development, the blots were washed three times for 10 minutes with TBS-T or TBS for Gγ blots. All blot were developed at 4°C. Blots were developed using chemiluminescent ECL reagents and detected on a Storm860 phosphoimager and quantified using ImageQuant software (GE Healthcare) or blots were developed using 4-chloronaphtol for colorimetric detection, the blot was photographed and digitized with an Alpha Innotech IS-500 digital imaging system, and the image was quantified using ImageJ software.

Antibody dilutions for immunoblotting were as follows. For PhLP-myc and Pdc-myc detection, the mouse anti-myc primary antibody was diluted 1:1000 and the goat anti-mouse horseradish peroxidase-conjugated secondary antibody was diluted 1:2000. For Flag-Gβ detection, the mouse anti-Flag primary antibody was diluted 1:1000 and the goat anti-mouse horseradish peroxidase-conjugated secondary antibody was diluted 1:2000. Endogenous PhLP was detected with a 1:5000 dilution of an anti-PhLP antibody (Thulin et al. 1999) followed by a 1:2000 dilution of goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody. Endogenous Gβ was detected with a 1:1500 dilution of affinity-purified anti-Gβ antibody (Lukov et al. 2004) incubated overnight and
then detected with a 1:2000 dilution of goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody. For HA-Gγ, a 1:500 dilution of rat anti-HA antibody and a 1:2000 dilution of goat-anti-rat horseradish peroxidase-conjugated secondary antibody were used.

Radiolabel Pulse-Chase Assay – The rate of release of nascent Gβ from CCT was measured using a radiolabel pulse-chase assay (Lukov et al., 2005). Six-well plates of HEK-293 cells were co-transfected with 1.0 μg of Flag Gβ, HA-Gγ or PhLP-myc cDNA variants as indicated. After a 10 min pulse, the radiolabel was chased 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 (Serotec) 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 (Lukov et al., 2005). The Gβ band was clearly separated from the other radiolabeled bands, facilitating its quantification. The amount of Gβ in the CCT immunoprecipitate was divided by that in the Flag-Gβ immunoprecipitate to determine the fraction of the total Gβ bound to CCT. These values were expressed as a percentage of the 30 min time point in order 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 $t_{1/2} = \ln 2/k$. 
CHAPTER 3
RESULTS

Gβ binds CCT in a ternary complex with PhLP – There appear to be inconsistencies, as noted in the introduction, concerning the role of the PhLP-CCT interaction in Gβγ assembly. The fact that the PhLP Δ1-75 variant with poor Gβγ binding but normal CCT binding was such a potent inhibitor of Gβγ assembly confirmed the requirement for PhLP in the formation of the Gβγ dimer (Lukov et al. 2005). In contrast, the lack of effect of the PhLP 132-135A variant with normal Gβγ binding but poor CCT binding brought into question the potential role of CCT in the assembly process. Yet the manner in which PhLP bound CCT above the folding cavity in the cryo-EM structure of the PhLP-CCT complex suggested that Gβ or Gβγ might sit below PhLP in the folding cavity, forming a ternary complex with CCT (Martin-Benito et al. 2004). Furthermore, the correlation between the increase in binding of PhLP to CCT upon phosphorylation of S18-20 and the necessity of phosphorylation of S18-20 for full activity in Gβγ assembly suggested that the effects of PhLP phosphorylation on assembly may occur through CCT. These apparently conflicting results led to further investigation of the potential role of CCT in Gβγ assembly.

If CCT does participate in the assembly process, then it must interact with Gβ or 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 over-expressed Gβ or Gγ in HEK-293 cells. Gβ co-immunoprecipitated with CCT.
robustly, to a similar extent as over-expressed PhLP, whereas over-expressed Pdc, which does not bind CCT, was not found in the CCT immunoprecipitate (Figure 3-1). Thus, Gβ appears to be specifically interacting with CCT under over-expression conditions. In contrast, over-expressed Gγ did not co-immunoprecipitate with CCT (Figure 3-1). To determine whether the interaction also occurred with endogenous amounts of Gβ, the experiment was also done without over-expressing Gβ. Co-immunoprecipitation of Gβ with CCT was also observed with endogenous Gβ, confirming the results of the over-expression experiments (Figure 3-2).

The manner in which PhLP binds CCT at the top of the apical domains without entering the folding cavity (Martin-Benito et al., 2004) 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 over-expression on the binding of the other to CCT was measured. As predicted, Gβ over-expression increased the binding of endogenous PhLP to CCT (Figure 3-3). However, PhLP over-expression unexpectedly caused a small but reproducible decrease in Gβ binding to CCT (Figure 3-2).

![Figure 3-1. Gβ binds CCT.](image)

**Figure 3-1. Gβ binds CCT.** HEK-293 cells were transfected with Flag-Gβ1, PhLP, Pdc or HA-Gγ2 and cell extracts were immunoprecipitated with an antibody to CCTε to bring down CCT complexes. The immunoprecipitates were immunoblotted for Gβ1, PhLP, Pdc or Gγ2.
Figure 3-2. CK2 phosphorylated PhLP mediates the release of Gβ from CCT. The effects of PhLP on Gβ binding to CCT measured by co-immunoprecipitation. HEK-293 cells were transfected with wild-type PhLP, the PhLP 218-20A or Δ1-75 variants, or empty vector. Cell extracts were immunoprecipitated with the anti-CCTe antibody and immunoblotted for endogenous Gβ1. A representative immunoblot is shown. Bars in the graph represent the average ± s.e. of the Gβ band intensity relative to the empty vector control from 4 separate experiments.

Figure 3-3. Gβ enhances the binding of PhLP to CCT. The effects of Gβ on the binding of endogenously expressed PhLP to CCT measured by co-immunoprecipitation. HEK-293 cells were transfected with Gβ1, CCT was immunoprecipitated as in Figure 3-2, and samples were immunoblotted for endogenous PhLP. A representative immunoblot is shown. Bars in the graph represent the average ± s.e. of the PhLP band intensity relative to the empty vector control from 3 separate experiments.
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 S18-20A and the other was a truncation variant in which residues 1-75 had been removed (PhLP Δ1-75) (Lukov et al., 2005). Both of these variants bind CCT, but they block Gβγ assembly in a dominant negative manner (Lukov et al., 2005). Over-expression of either of these variants increased endogenous Gβ binding to CCT dramatically (Figure 3-2). Thus, it appears that in the absence of S18-20 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 (Lukov et al., 2005), 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 over-expressed in HEK-293 cells with Gγ and PhLP variants as indicated, and the amount of Gβ co-immunoprecipitating with CCT was measured (Figure 3-4). Co-expression of Gγ caused a decrease in Gβ binding to CCT that was intensified by the co-expression of 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 S18-20A 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 S18-20A 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 (Lukov et al., 2005) and Gγ-mediated dissociation of Gβ from CCT, while PhLP S18-20A decreased the rate of Gβγ assembly by 15-fold (Lukov et al., 2005) and Gγ-induced dissociation of Gβ from CCT by 9-fold (compare the Gβγ PhLP-WT sample to the Gβγ PhLP S18-20A sample in Figure 3-4). 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.

**Figure 3-4.** The effects of PhLP phosphorylation and Gγ co-expression on Gβ binding to CCT. HEK-293 cells were transfected with Flag-Gβ1, HA-Gγ2 and the PhLP variants as indicated. Cell extracts were immunoprecipitated with an antibody to CCTε and then immunoblotted for 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 band from 3 separate experiments.
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 over-expression on the rate of dissociation of Gβ from CCT were measured. In this experiment, cells co-expressing Gβ with Gγ, PhLP or PhLP S18-20A were pulsed with ^35S-methionine for 10 minutes 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] 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 t_{1/2} of ~ 8 hrs. PhLP co-expression increased the rate by 4-fold to a t_{1/2} of ~ 2 hrs. In contrast, PhLP S18-20A co-expression did not increase the dissociation rate (Figure 3-5A). When Gγ was co-expressed with Gβ, the dissociation rate increased by more than 2-fold to a t_{1/2} of ~ 3 hrs, while when both Gγ and PhLP were co-expressed, the t_{1/2} increased even further to ~ 2 hrs, the same value observed in the absence of Gγ over-expression (Figure 3-5B). When PhLP S18-20A was co-expressed with Gγ, there was essentially no Gβ dissociation, similar to what was seen in the absence of Gγ over-expression (Figure 3-5B). These effects of Gγ, PhLP and PhLP
S18-20A on the dissociation rates are consistent with their effect on the steady-state binding of Gβ to CCT (Figure 3-4) 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 proposed above. The enhanced rate of dissociation of Gβ from CCT upon PhLP over-expression in the absence of Gγ over-expression (Figure 3-5A) is consistent with the second mechanism in which a phosphorylated PhLP-Gβ complex would be released prior to Gγ binding to Gβ.

**Table 3.5.** The effects of PhLP phosphorylation and Gγ on the rate of Gβ release from CCT. (A) HEK-293 cells were transfected with Flag-Gβ1 and the indicated PhLP variants. The pulse-chase assay was performed with times indicating the sum of the 10 min pulse and the variable chase periods. After the chase times indicated, cell extracts were immunoprecipitated with antibodies to CCTe or Gβ1. Proteins were resolved by SDS-PAGE and radiolabeled bands were detected using a phosphorimager. The Gβ band intensities were quantified, and ratios of nascent Gβ1 in the CCT immunoprecipitate versus the total nascent Gβ in the Gβ immunoprecipitate were calculated and plotted as a percentage of the ratio at the first time point. Lines represent a fit of the data from 3 separate experiments to a first-order rate equation. Values for t1/2 are shown below the graph. (B) The effects of Gγ on PhLP-mediated release of nascent Gβ1 from CCT were measured as in panel A in HEK-293 cells co-expressing HA-Gγ2 in addition to Flag-Gβ1 and PhLP.
This result would not be expected in the first mechanism in which G\(\gamma\) binding would be required for release of G\(\beta\) from CCT. Similarly, the observed lack of increase in the G\(\beta\) dissociation rate upon co-expression of G\(\gamma\) with PhLP would be predicted by the second mechanism but not by the first. On the other hand, the increased release of G\(\beta\) from CCT upon G\(\gamma\) over-expression in the absence of PhLP over-expression 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 G\(\beta\) from CCT for association with G\(\gamma\). In this case, the dissociation process would be drawn forward by the formation of the G\(\beta\)\(\gamma\) dimer and its association with G\(\alpha\) and cell membranes (see Figure 3-7).

To further assess the role of G\(\gamma\) in the release of G\(\beta\) from CCT, the possible association of G\(\gamma\) with G\(\beta\) and PhLP in CCT complexes was determined. G\(\gamma\) was co-expressed with the indicated combinations of G\(\beta\) and the PhLP variants, the CCT complexes were immunoprecipitated and the samples were immunoblotted for G\(\gamma\). G\(\gamma\) was not found in any of the CCT immunoprecipitates (Figure 3-6), despite the fact that G\(\beta\) and PhLP could be readily found under these conditions (see Figures 3-1 – 3-3). Thus, it appears that G\(\gamma\) does not interact with CCT in any of its complexes with G\(\beta\) and PhLP.

![Figure 3-6. Assessment of G\(\gamma\) binding to CCT.](image-url)

HEK-293 cells were transfected with Flag-G\(\beta_1\), HA-G\(\gamma\), or PhLP variants as indicated. Cells extracts were immunoprecipitated with an antibody to CCT\(\varepsilon\) and then immunoblotted for G\(\gamma\). A representative blot is shown. The Std lane in the CCT IP was lysate from the G\(\gamma\) transfected cells.
Together, the data in Figures 3-4 thru 3-6 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 (Lukov et al., 2005).

**Figure 3-7. CK2 phosphorylation-dependent release model of Gβγ assembly.** A model is proposed in which nascent Gβ forms a ternary complex with CCT and PhLP. If PhLP is not phosphorylated, the ternary complex is stable and PhLP-Gβ is not released from CCT. If PhLP is phosphorylated, the ternary complex is destabilized, possibly by electrostatic repulsion between the phosphates in the S18-20 phosphorylation site and negatively charged residues on the CCTα or ε apical domains. Once released, the PhLP-Gβ complex binds Gγ, forming the Gβγ dimer. The dimer then associates with Gα and 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 S18-20 phosphorylation site is depicted by a red oval marked (P). The relative amount of positive and negative charge on the CCT apical domains that contact the PhLP N-terminal domain is also indicated. See text for details.
CHAPTER 4
DISCUSSION

A model for G\(\beta\gamma\) assembly – Recent studies have shown that PhLP acts as an essential chaperone in the assembly of G\(\beta\gamma\) dimers by binding the G\(\beta\) subunit and thereby allowing G\(\gamma\) to associate with G\(\beta\) (Lukov et al., 2005; Humrich et al., 2005). Phosphorylation of PhLP at S18-20 by CK2 was required for G\(\beta\gamma\) assembly to occur, yet the means by which S18-20 phosphorylation contributes to assembly was unknown. Moreover, CCT had been implicated in the assembly process, but the results were conflicting (Martin-Benito et al., 2004; Lukov et al., 2005; Humrich et al., 2005). The current study provides evidence for a molecular mechanism describing both the role of CCT and PhLP phosphorylation in G\(\beta\gamma\) assembly (Figure 3-7). 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 forms in a stable conformation that does not release PhLP-G\(\beta\) and the G\(\beta\gamma\) assembly process is blocked. However, if PhLP is phosphorylated within the S18-20 sequence, 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\(\beta\gamma\) complex shows that Pdc binds G\(\beta\) on the opposite face as G\(\gamma\) (Gaudet et al., 2006), 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 (Savage et al., 2000). However, both the G\(\alpha\) binding site and the membrane association surface of G\(\beta\gamma\) overlap.
extensively with the PhLP binding site (Savage et al., 2000). Gα and PhLP share similar binding affinities for Gβγ. However, significantly more Gα is present in the cell, and there is an abundance of membrane surface on the endoplasmic reticulum and Golgi for Gβγ binding. Therefore, in the cell PhLP would be expected to be released from Gβγ. 5) Gβγ associates with Gα and/or the endoplasmic reticulum membrane and is transported to the plasma membrane (Michaelson et al., 2002). PhLP is then free to catalyze another round of Gβγ assembly.

This model readily explains the dominant negative effect of the PhLP S18-20A and PhLP Δ1-75 variants. These variants form PhLP-Gβ-CCT ternary complexes that do not release PhLP-Gβ for Gγ binding. Such stable ternary complexes would also block the endogenous, phosphorylated PhLP from forming competent ternary complexes capable of releasing PhLP-Gβ for Gγ binding. Previous explanations of the dominant negative effect of PhLP S18-20A which postulated that unphosphorylated PhLP would block Gβ and Gγ association with CCT (Humrich et al., 2005) or that unphosphorylated PhLP would form a PhLP-Gβ complex that would not accept Gγ (Lukov et al., 2005) are incomplete.

*Phosphorylation-induced conformational changes* – Although CK2 phosphorylation increases PhLP’s binding to CCT in the absence of Gβ (Lukov et al., 2006), PhLP phosphorylation was necessary for the release of PhLP-Gβ from CCT in the presence of Gβ (Figures. 3-4,5). 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 cryo-EM studies of the unphosphorylated PhLP-CCT complex (Martin-Benito et al., 2004). 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 (Martin-Benito et al., 2004). In one conformation, the N-terminal phosphorylation site of PhLP was in close proximity to the CCT\(\alpha\) and \(\epsilon\) apical domains and in the other conformation the phosphorylation site was in close proximity to the CCT\(\zeta\) and \(\beta\) apical domains. The binding surfaces of all eight apical domains are dominated by charged and polar residues (Pappenberger et al., 2002) with the CCT\(\alpha\) and \(\epsilon\) binding surfaces having a high distribution of negative charge, while the CCT\(\zeta\) binding surface exhibits an extensive positively charged patch.

The S18-20 phosphorylation site of PhLP is harbored within a sequence (S\(_{18}\)SSDEDESD) 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 CCT\(\zeta\). In the absence of G\(\beta\), phosphorylation could favor the conformation that brings the PhLP phosphorylation site in close proximity to the CCT\(\zeta\) apical domain, increasing the binding of PhLP to CCT. In the presence of G\(\beta\), it is possible that interactions with G\(\beta\) 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 CCT\(\alpha\) and \(\epsilon\) apical domains, causing electrostatic repulsion between the negative charges on the CCT\(\alpha\) and \(\epsilon\) binding surfaces and the PhLP phosphorylation site. This repulsion might destabilize the ternary complex and allow the release of the PhLP-G\(\beta\) complex. Further studies will be required
to test the validity of this structural model. It is not clear from the current data whether phosphorylation occurs prior to or after association of PhLP with CCT (Figure 3-7). These investigations into the mechanism of PhLP-mediated Gβγ assembly and its regulation by CK2 phosphorylation suggest that PhLP’s interactions with Gβ 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 such as heart disease, developmental, immunological, gastrointestinal and psychological disorders, addictions, and certain forms of cancer (Wettschureck and Offermanns 2005).


