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**THE MECHANISM OF ASSEMBLY OF THE G-PROTEIN  $\beta\gamma$   
SUBUNIT DIMER BY CK2 PHOSPHORYLATED PHOSDUCIN-  
LIKE PROTEIN AND THE CHAPERONIN CONTAINING TCP-1**

by

Christine Baker

A thesis submitted to the faculty of  
Brigham Young University  
in partial fulfillment of the requirements for the degree of  
Masters of Science

Department of Chemistry and Biochemistry  
Brigham Young University

May 2006

BRIGHAM YOUNG UNIVERSITY

**GRADUATE COMMITTEE APPROVAL**

of a thesis submitted by

Christine Baker

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the thesis of Christine Baker in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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## ABSTRACT

### THE MECHANISM OF ASSEMBLY OF THE G-PROTEIN $\beta\gamma$ SUBUNIT DIMER BY CK2 PHOSPHORYLATED PHOSDUCIN-LIKE PROTEIN AND THE CHAPERONIN CONTAINING TCP-1

Phosducin-like protein (PhLP) binds G-protein  $\beta\gamma$  subunits and is thought to assist in assembly of the  $G\beta\gamma$  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  $G\beta\gamma$  dimer formation remains unclear. This study addresses the mechanism of  $G\beta\gamma$  assembly by exploring the specific roles of CCT and CK2 phosphorylation of PhLP in the assembly process. Both overexpressed and endogenous  $G\beta$  were shown to co-immunoprecipitate with CCT to a similar extent as PhLP, indicating that CCT may be involved in the folding of  $G\beta$ . In addition,  $G\beta$  overexpression enhanced the binding of PhLP to CCT, suggesting the formation of a ternary PhLP- $G\beta$ -CCT complex. In contrast, overexpression of PhLP caused the release of  $G\beta$  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  $G\beta\gamma$  dimer formation, suggesting a correlation between PhLP-mediated release of  $G\beta$  from CCT and  $G\beta\gamma$  assembly. Experiments investigating the role of  $G\gamma$  in this process show that  $G\gamma$  does not interact with CCT nor is it the essential factor in the release of  $G\beta$  from CCT. A new model is therefore proposed for  $G\beta\gamma$  assembly

involving the formation of a PhLP-G $\beta$ -CCT ternary complex followed by the release of a phosphorylated PhLP-G $\beta$  complex from CCT. In the PhLP-G $\beta$  complex, the G $\gamma$  binding face of G $\beta$  is exposed, allowing for the formation of the G $\beta\gamma$  dimer.

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## ABBREVIATIONS

AC	Adenylyl cyclase
CCT	<u>Ch</u> aperonin <u>C</u> ontaining <u>T</u> ailless complex polypeptide 1 (TCP-1)
CHO cells	Chinese hamster ovary cells
CK2	Casein kinase 2
Cryo-EM	Cryoelectron microscopy
G $\alpha$	G-protein alpha subunit
G $\beta$	G-protein beta subunit
G $\beta\gamma$	G-protein beta and gamma subunit dimer
G $\gamma$	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

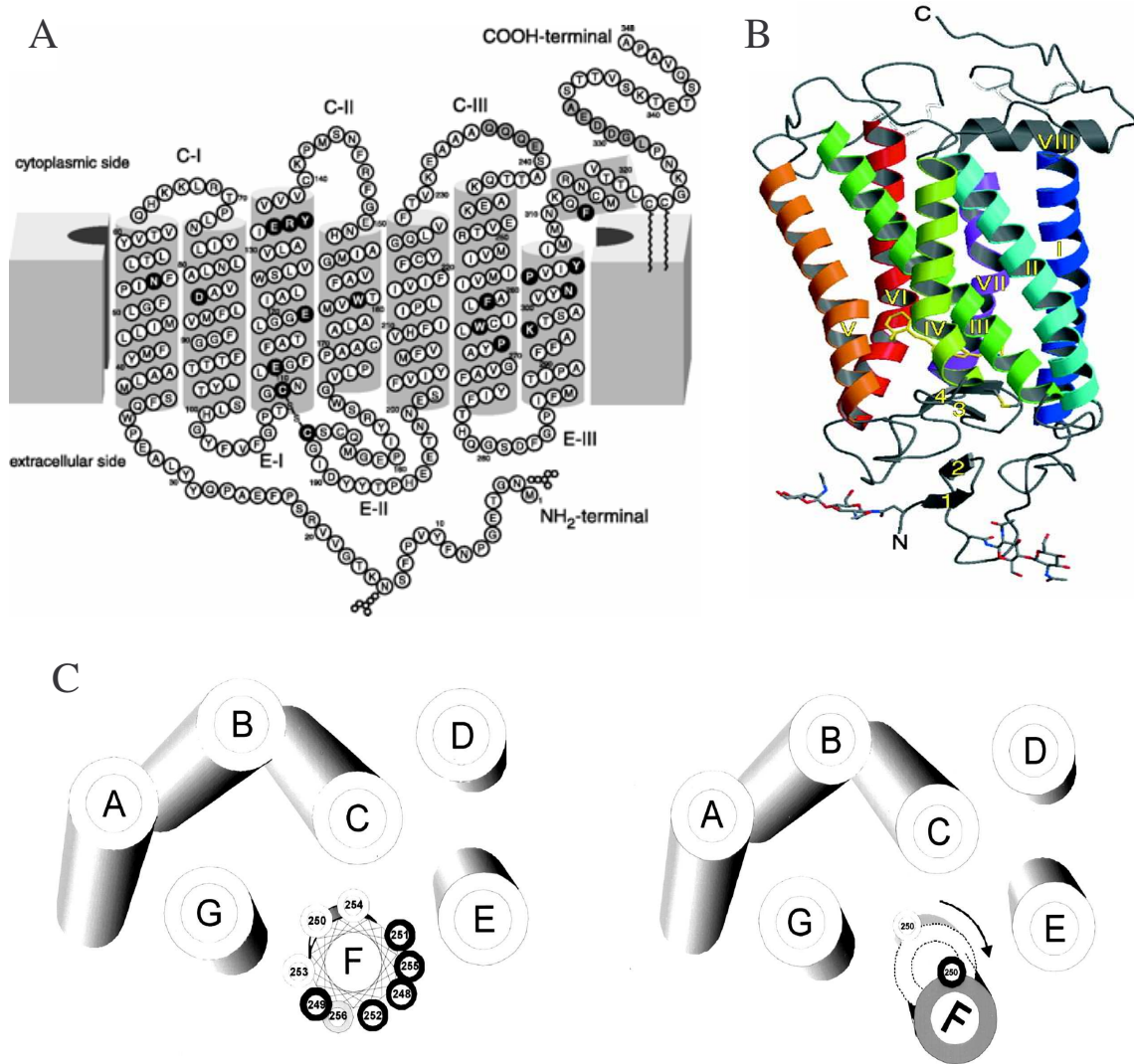
### INTRODUCTION TO G-PROTEIN SIGNALING, PHOSDUCIN-LIKE PROTEINS, AND THE CYTOSOLIC CHAPERONIN CONTAINING TCP-1

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; Sealson, 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  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits that associate with the intracellular surface of the plasma membrane. Binding to an activated receptor causes the dissociation of GDP from  $G\alpha$ , allowing GTP to enter in its place in the nucleotide binding site. GTP binding causes a conformational change in the  $G\alpha$  subunit, destabilizing its interaction with  $G\beta\gamma$ , resulting in the dissociation of  $G\alpha$ -GTP from  $G\beta\gamma$ . 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  $G\alpha$  subunit, making it available to reassociate with  $G\beta\gamma$  and return to the plasma membrane to await activation once again.

Although  $G\alpha$  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  $G\alpha$ 's hydrolysis of GTP to GDP. RGS proteins act by stabilizing  $G\alpha$  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  $G\alpha$  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 G $\alpha$  subunits can be grouped into four main families—G $\alpha_s$ , G $\alpha_{q/11}$ , G $\alpha_{12/13}$ , and G $\alpha_{i/o}$ , with each family containing several genes that target similar effectors. G $\alpha_s$  primarily activates isoforms of adenylyl cyclase (AC) while G $\alpha_{q/11}$  stimulates isoforms of phospholipase C- $\beta$  (PLC- $\beta$ ). G $\alpha_{12/13}$  has a large number of effectors varying from Na<sup>+</sup>/H<sup>+</sup> exchangers to RhoA GTPase activating proteins. G $\alpha_{i/o}$  family members also have a variety of effectors with inhibition of AC and activation of cGMP phosphodiesterase as the principle targets. Because G $\alpha_{i/o}$  is often found in higher concentrations, its dissociation from its G $\beta\gamma$  subunits is thought to allow G $\beta\gamma$  to activate its downstream effectors (Wettschureck and Offermanns, 2005).

Originally, G $\beta\gamma$  was thought to lack its own effectors and only facilitate G-protein signaling by assisting G $\alpha$ 's interaction with the plasma membrane and receptors. However, the existence of five different G $\beta$  subunits and twelve G $\gamma$  subunits in the human genome suggest this first hypothesis is unlikely (Cabrera-Vera et al., 2005; Wettschureck and Offermanns, 2005). Four of the five G $\beta$  subunits bind G $\gamma$  tightly and these G $\beta\gamma$  complexes have been shown more recently to have a number of effectors. Note that G $\beta_5$  has less sequence homology than the other four G $\beta$  subunits and binds G $\gamma$  weakly. Although G $\beta_5$  has some *in vitro* interactions with the G $\gamma_2$ , it displays greater affinity for the G $\gamma$ -like domain of RGS proteins, suggesting a slightly different contribution to G-protein signaling (Yoshikawa et al., 2000; Wettschureck and Offermanns, 2005). The other G $\beta\gamma$  dimers are known to upregulate isoforms of PLC- $\beta$  and the  $\beta$ -adrenergic receptor kinase and to downregulate isoforms of AC (Cabrera-Vera et al., 2003; Wettschureck and Offermanns, 2005). G $\beta\gamma$  also regulates ion-channels,

activating G-protein-activated inwardly rectifying K<sup>+</sup> channels (GIRKs) and inhibiting voltage-gated Ca<sup>2+</sup> channels (Dascal, 2001). As the number of known Gβγ effectors continues to grow, it becomes increasingly important to understand the regulation of these subunits.

Two Gβγ binding partners have been described as regulators of Gβγ 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 Gβγ with high affinity (Savage et al., 2000). PhLP isoforms in subgroups II and III do not bind Gβγ, and all future references to PhLP will refer to the member of the Pdc-I family.

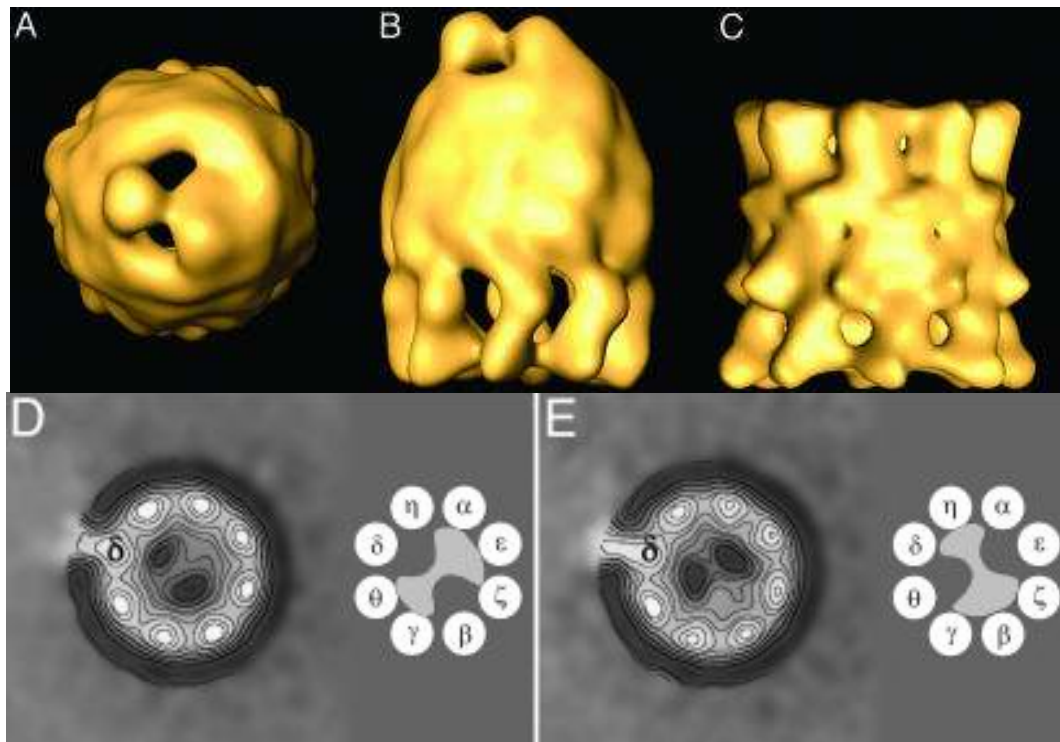
PhLP shares a 41% amino acid identity and a 65% homology with Pdc (Miles et al. 1993). Both Pdc and PhLP's interactions with the Gβγ were initially thought to be inhibitory in nature, preventing Gβγ from performing its normal functions (Bauer et al., 1992; Lee et al., 1992; Schroder and Lohse, 1996; 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 Gβ 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  $\alpha/\epsilon/\zeta$  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 G $\beta$  $\gamma$  dimer assembly (Lukov et al., 2005). PhLP knockdown in mammalian cells decreased the total G $\beta$  protein present in the cell without changing the G $\beta$  mRNA level. This result suggested a translational or post-translational affect of PhLP on G $\beta$ . Further investigation showed that the ability of G $\beta$  to assemble into dimers with G $\gamma$  was impaired upon siRNA-mediated depletion of PhLP. The rate at which G $\beta$  $\gamma$  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 G $\beta$  $\gamma$  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 *in vitro* and *in vivo* and appear to contribute to PhLP's regulation of G $\beta$  $\gamma$  (Humrich, et al., 2003; Lukov et al., 2006). Overexpression of PhLP mutant S18-20A, which cannot be phosphorylated at those residues, decreases G $\beta$  $\gamma$  expression by 70-80%. Furthermore, PhLP S18-20A overexpression decreased the rate of G $\beta$  $\gamma$  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 G $\beta$  $\gamma$ . Note that another study shows that CK2 phosphorylation at these residues appears to have no affect on PhLP binding to G $\beta$  $\gamma$ , 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  $\Delta$ 1-75) binds G $\beta$  with low affinity but binds CCT well. This variant was even more effective in inhibiting G $\beta\gamma$  expression and assembly than the PhLP S18-20A variant. Furthermore, another PhLP variant (PhLP 132-135A) that binds G $\beta$  normally but CCT very poorly shows no negative affect on the rate of G $\beta\gamma$  assembly compared to PhLP-WT (Lukov et al., 2005).

Clearly the roles of PhLP-CCT and PhLP-G $\beta\gamma$  interactions in G $\beta\gamma$  assembly remain inconclusive, particularly in relationship to each other. From these studies it appears that the PhLP-CCT interaction is not necessary for G $\beta\gamma$  assembly while the PhLP-G $\beta\gamma$  interaction and CK2 phosphorylation of PhLP are essential. In contrast, another study shows that siRNA-mediated depletion of CCT leads to a decrease in G $\beta\gamma$  expression, a similar affect to that of the PhLP S18-20A and PhLP  $\Delta$ 1-75 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 G $\beta\gamma$  assembly process by showing the formation of a ternary PhLP-G $\beta$ -CCT complex. Furthermore, the role of PhLP phosphorylation in this process is shown to mediate the release of G $\beta$  from CCT thereby allowing it to interact with G $\gamma$ . A new model is therefore proposed for the assembly of G $\beta\gamma$  through the formation of a ternary PhLP-G $\beta$ -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 [<sup>35</sup>S] Methionine were from Amersham Biosciences. The anti-Gβ<sub>1</sub> 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-ε (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% CO<sub>2</sub> 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<sub>6</sub> tags (Invitrogen) as described (Carter et al., 2004;

Lukov et al., 2005). PhLP variants S18-20A and  $\Delta$ 1-75 were both created using PCR based methods as described (Lukov, 2005). N-terminal HA-tagged  $G\gamma_2$  and N-terminal Flag-tagged  $G\beta_1$  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  $G\beta$  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  $\mu$ l of immunoprecipitation buffer (~2% IGEPAL (NP-40) in PBS) per well or 1.2 ml per dish. For each ml of this solution, 6  $\mu$ l of 100 mM PMSF and 4.5  $\mu$ l 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  $\mu$ l of sample was removed and diluted in 40  $\mu$ l  $H_2O$  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  $\mu$ g anti-CCT $\epsilon$  per well, 3.5  $\mu$ g per 2-wells for endogenous PhLP, or 10  $\mu$ g per 100 mm dish for endogenous  $G\beta$  and incubated with rotation at 4°C for at least 30 minutes. This was followed by the addition of 25, 35, or 60  $\mu$ l 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  $G\beta$

samples which were not boiled, and then resolved on 10% Tris-Glycine SDS-PAGE or 16.5% Tris-Tricine SDS-PAGE gels for G $\gamma$  detection.

Proteins were transferred to a nitrocellulose membrane and blots were blocked in 5% non-fat dry milk in TBS-T. Anti-HA G $\gamma$  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 G $\gamma$  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-chloronaphthol 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-G $\beta$  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 G $\beta$  was detected with a 1:1500 dilution of affinity-purified anti-G $\beta$  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-G $\gamma_2$ , 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 G $\beta$  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  $\mu\text{g}$  of Flag G $\beta$ , HA-G $\gamma$  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  $\mu\text{l}$  of immunoprecipitation buffer. The extract was divided into two 95  $\mu\text{l}$  samples and 2.5  $\mu\text{l}$  of 1  $\mu\text{g}/\mu\text{l}$  anti-CCT $\epsilon$  antibody (Serotec) was added to one sample and 3.0  $\mu\text{l}$  of 1  $\mu\text{g}/\mu\text{l}$  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 G $\beta$  band was clearly separated from the other radiolabeled bands, facilitating its quantification. The amount of G $\beta$  in the CCT immunoprecipitate was divided by that in the Flag-G $\beta$  immunoprecipitate to determine the fraction of the total G $\beta$  bound to CCT. These values were expressed as a percentage of the 30 min time point in order to readily compare the rates of G $\beta$  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

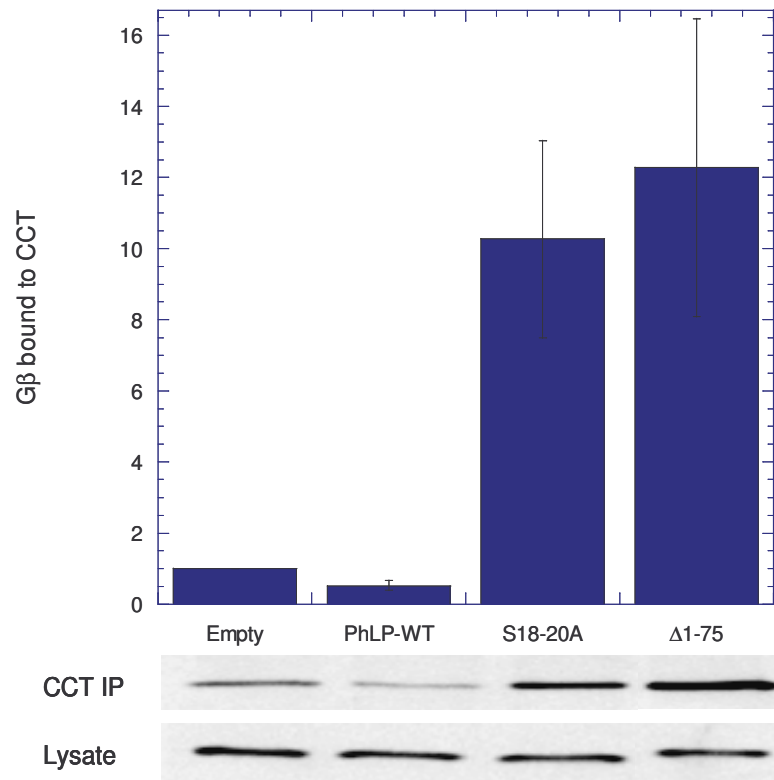


robustly, to a similar extent as over-expressed PhLP, whereas over-expressed Pdc, which does not bind CCT, was not found in the CCT immunoprecipitate (Figure 3-1). Thus, G $\beta$  appears to be specifically interacting with CCT under over-expression conditions. In contrast, over-expressed G $\gamma$  did not co-immunoprecipitate with CCT (Figure 3-1). To determine whether the interaction also occurred with endogenous amounts of G $\beta$ , the experiment was also done without over-expressing G $\beta$ . Co-immunoprecipitation of G $\beta$  with CCT was also observed with endogenous G $\beta$ , confirming the results of the over-expression experiments (Figure 3-2).

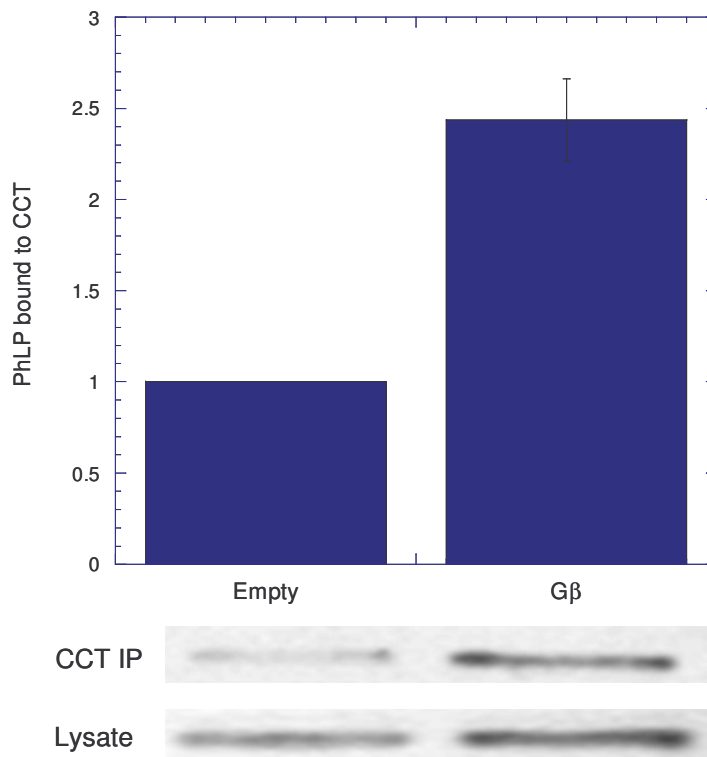
The manner in which PhLP binds CCT at the top of the apical domains without entering the folding cavity (Martin-Benito et al., 2004) suggests that PhLP, G $\beta$  and CCT might form a ternary complex in the process of G $\beta\gamma$  folding. If such a ternary complex does exist, then PhLP would be predicted to increase the binding of G $\beta$  to CCT and vice versa. To test this possibility, the effects of PhLP or G $\beta$  over-expression on the binding of the other to CCT was measured. As predicted, G $\beta$  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 G $\beta$  binding to CCT (Figure 3-2).



**Figure 3-1. G $\beta$  binds CCT.** HEK-293 cells were transfected with Flag-G $\beta_1$ , PhLP, Pdc or HA-G $\gamma_2$  and cell extracts were immunoprecipitated with an antibody to CCT $\epsilon$  to bring down CCT complexes. The immunoprecipitates were immunoblotted for G $\beta_1$ , PhLP, Pdc or G $\gamma_2$ .



**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-CCTε antibody and immunoblotted for endogenous Gβ<sub>1</sub>. 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.

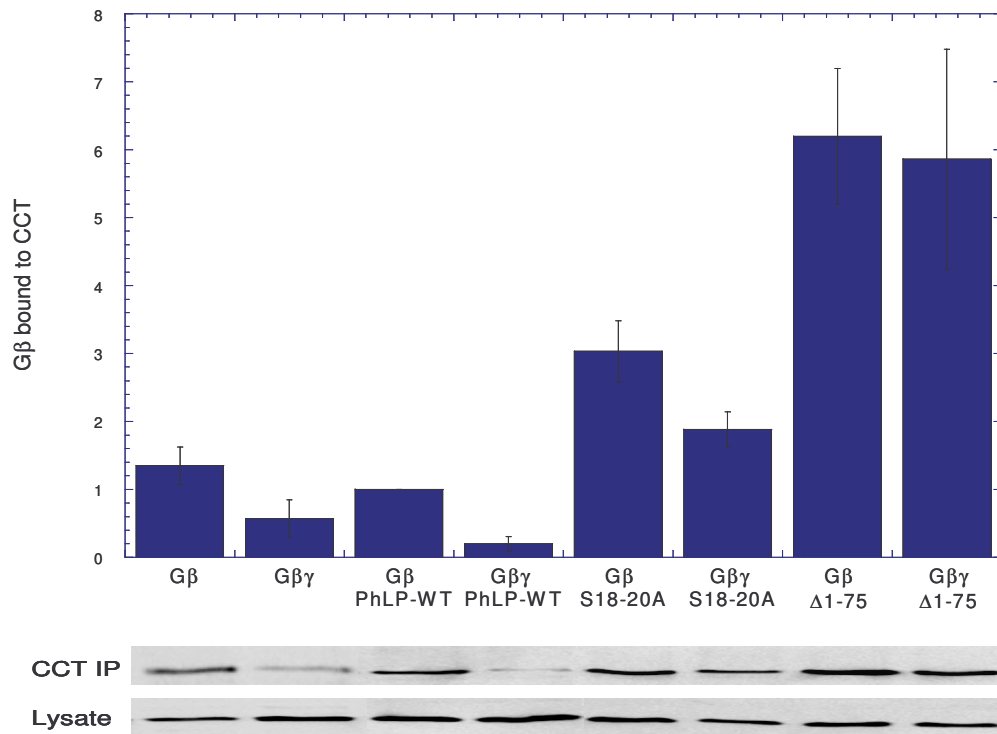


**Figure 3-3. Gβ enhances the binding of PhLP to CCT.** The effects of Gβ on the binding of endogenously expressed PhLP to CCT measured by co-immunoprecipitation. HEK-293 cells were transfected with Gβ<sub>1</sub>, CCT was immunoprecipitated as in Figure 3-2, and samples were immunoblotted for endogenous 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 G $\beta$  binding to CCT might be caused by PhLP catalyzed G $\beta\gamma$  assembly and release of the G $\beta\gamma$  dimer from CCT. To test this possibility, the effects of two PhLP variants that do not support G $\beta\gamma$  assembly on G $\beta$  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  $\Delta$ 1-75) (Lukov et al., 2005). Both of these variants bind CCT, but they block G $\beta\gamma$  assembly in a dominant negative manner (Lukov et al., 2005). Over-expression of either of these variants increased endogenous G $\beta$  binding to CCT dramatically (Figure 3-2). Thus, it appears that in the absence of S18-20 phosphorylation, PhLP forms a ternary complex with G $\beta$  and CCT that cannot progress in the assembly process. It is interesting to note that the PhLP $\Delta$ 1-75 variant binds G $\beta\gamma$  very poorly (Lukov et al., 2005), yet it is still able to stabilize the complex between G $\beta$  and CCT. This observation indicates that PhLP  $\Delta$ 1-75 may do so more through interactions with CCT than through interactions with G $\beta$ .

*PhLP phosphorylation is required for the release of G $\beta$  from CCT and interaction with G $\gamma$*  - To further investigate the apparent correlation between the destabilization of the PhLP-G $\beta$ -CCT ternary complex by PhLP phosphorylation and the requirement for PhLP phosphorylation in G $\beta\gamma$  assembly, the effects of G $\gamma$  on ternary complex formation with several PhLP variants was measured. G $\beta$  was over-expressed in HEK-293 cells with G $\gamma$  and PhLP variants as indicated, and the amount of G $\beta$  co-immunoprecipitating with CCT was measured (Figure 3-4). Co-expression of G $\gamma$  caused a decrease in G $\beta$  binding to CCT that was intensified by the co-expression of wild-type PhLP. In striking contrast, G $\beta$  binding to CCT was greatly enhanced by co-expression of PhLP  $\Delta$ 1-75 and

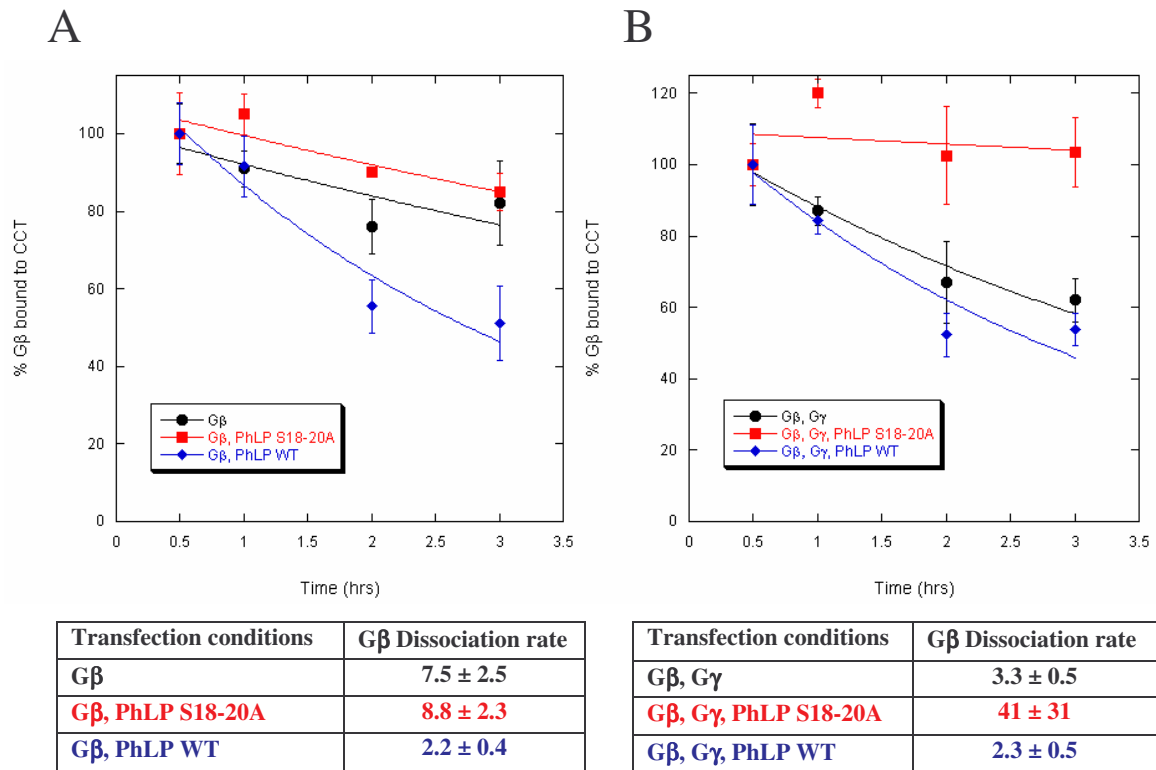
was completely insensitive to co-expression of  $G\gamma$ . Co-expression of PhLP S18-20A also enhanced  $G\beta$  binding to CCT significantly, and  $G\gamma$  had much less of an effect on binding than with wild-type PhLP. Interestingly, the effects of PhLP  $\Delta 1-75$  and S18-20A on  $G\beta$  binding to CCT in the presence of  $G\gamma$  were quantitatively very similar to their effects on  $G\beta\gamma$  assembly. PhLP  $\Delta 1-75$  completely blocked  $G\beta\gamma$  assembly (Lukov et al., 2005) and  $G\gamma$ -mediated dissociation of  $G\beta$  from CCT, while PhLP S18-20A decreased the rate of  $G\beta\gamma$  assembly by 15-fold (Lukov et al., 2005) and  $G\gamma$ -induced dissociation of  $G\beta$  from CCT by 9-fold (compare the  $G\beta\gamma$  PhLP-WT sample to the  $G\beta\gamma$  PhLP S18-20A sample in Figure 3-4). From these data, it appears that PhLP phosphorylation contributes to  $G\beta\gamma$  assembly by enhancing the ability of  $G\gamma$  to release  $G\beta$  from the ternary complex.



**Figure 3-4. The effects of PhLP phosphorylation and  $G\gamma$  co-expression on  $G\beta$  binding to CCT.** HEK-293 cells were transfected with Flag- $G\beta_1$ , HA- $G\gamma_2$  and the PhLP variants as indicated. Cell extracts were immunoprecipitated with an antibody to CCT $\epsilon$  and then immunoblotted for  $G\beta$ . A representative immunoblot is shown. Bars in the graph represent the average  $\pm$  s.e. of the  $G\beta$  band intensity relative to the  $G\beta$  PhLP-WT band from 3 separate experiments.

There are two possible mechanisms by which phosphorylated PhLP could contribute to  $G\gamma$ -mediated release of  $G\beta$  from CCT. Both involve a conformational change in the ternary complex upon PhLP phosphorylation. First, PhLP phosphorylation could induce a conformation that allows  $G\gamma$  to access  $G\beta$  in the ternary complex and form the  $G\beta\gamma$  dimer. The  $G\beta\gamma$  would then be released from CCT. Second, phosphorylation could induce a conformation that releases PhLP- $G\beta$  from CCT, thereby freeing the  $G\gamma$  binding site on  $G\beta$  for  $G\beta\gamma$  association to occur. To distinguish between these two mechanisms, the effects of  $G\gamma$  and PhLP over-expression on the rate of dissociation of  $G\beta$  from CCT were measured. In this experiment, cells co-expressing  $G\beta$  with  $G\gamma$ , PhLP or PhLP S18-20A were pulsed with  $^{35}\text{S}$ -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 [ $^{35}\text{S}$ ] in the  $G\beta$  band was quantified. In the absence of PhLP or  $G\gamma$  co-expression, the dissociation rate of nascent  $G\beta$  from CCT was very slow, with a  $t_{1/2}$  of  $\sim 8$  hrs. PhLP co-expression increased the rate by 4-fold to a  $t_{1/2}$  of  $\sim 2$  hrs. In contrast, PhLP S18-20A co-expression did not increase the dissociation rate (Figure 3-5A). When  $G\gamma$  was co-expressed with  $G\beta$ , the dissociation rate increased by more than 2-fold to a  $t_{1/2}$  of  $\sim 3$  hrs, while when both  $G\gamma$  and PhLP were co-expressed, the  $t_{1/2}$  increased even further to  $\sim 2$  hrs, the same value observed in the absence of  $G\gamma$  over-expression (Figure 3-5B). When PhLP S18-20A was co-expressed with  $G\gamma$ , there was essentially no  $G\beta$  dissociation, similar to what was seen in the absence of  $G\gamma$  over-expression (Figure 3-5B). These effects of  $G\gamma$ , PhLP and PhLP

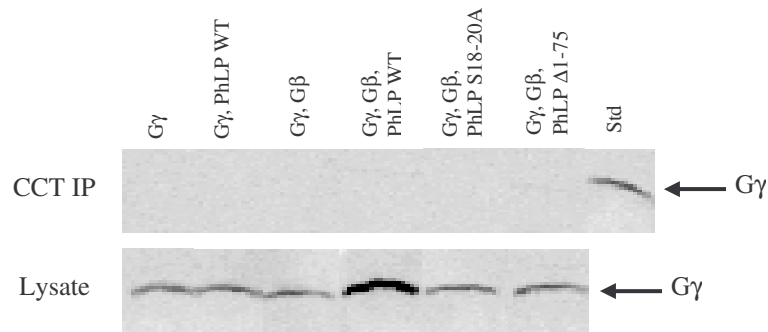
S18-20A on the dissociation rates are consistent with their effect on the steady-state binding of G $\beta$  to CCT (Figure 3-4) and further demonstrate that PhLP phosphorylation is required for the release of G $\beta$  from the ternary complex. These findings are able to distinguish between the two potential mechanisms proposed above. The enhanced rate of dissociation of G $\beta$  from CCT upon PhLP over-expression in the absence of G $\gamma$  over-expression (Figure 3-5A) is consistent with the second mechanism in which a phosphorylated PhLP-G $\beta$  complex would be released prior to G $\gamma$  binding to G $\beta$ .



**Figure 3-5. The effects of PhLP phosphorylation and G $\gamma$  on the rate of G $\beta$  release from CCT.** (A) HEK-293 cells were transfected with Flag-G $\beta_1$  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 CCT $\epsilon$  or G $\beta_1$ . Proteins were resolved by SDS-PAGE and radiolabeled bands were detected using a phosphorimager. The G $\beta$  band intensities were quantified, and ratios of nascent G $\beta_1$  in the CCT immunoprecipitate versus the total nascent G $\beta$  in the G $\beta$  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  $t_{1/2}$  are shown below the graph. (B) The effects of G $\gamma$  on PhLP-mediated release of nascent G $\beta_1$  from CCT were measured as in panel A in HEK-293 cells co-expressing HA-G $\gamma_2$  in addition to Flag-G $\beta_1$  and PhLP.

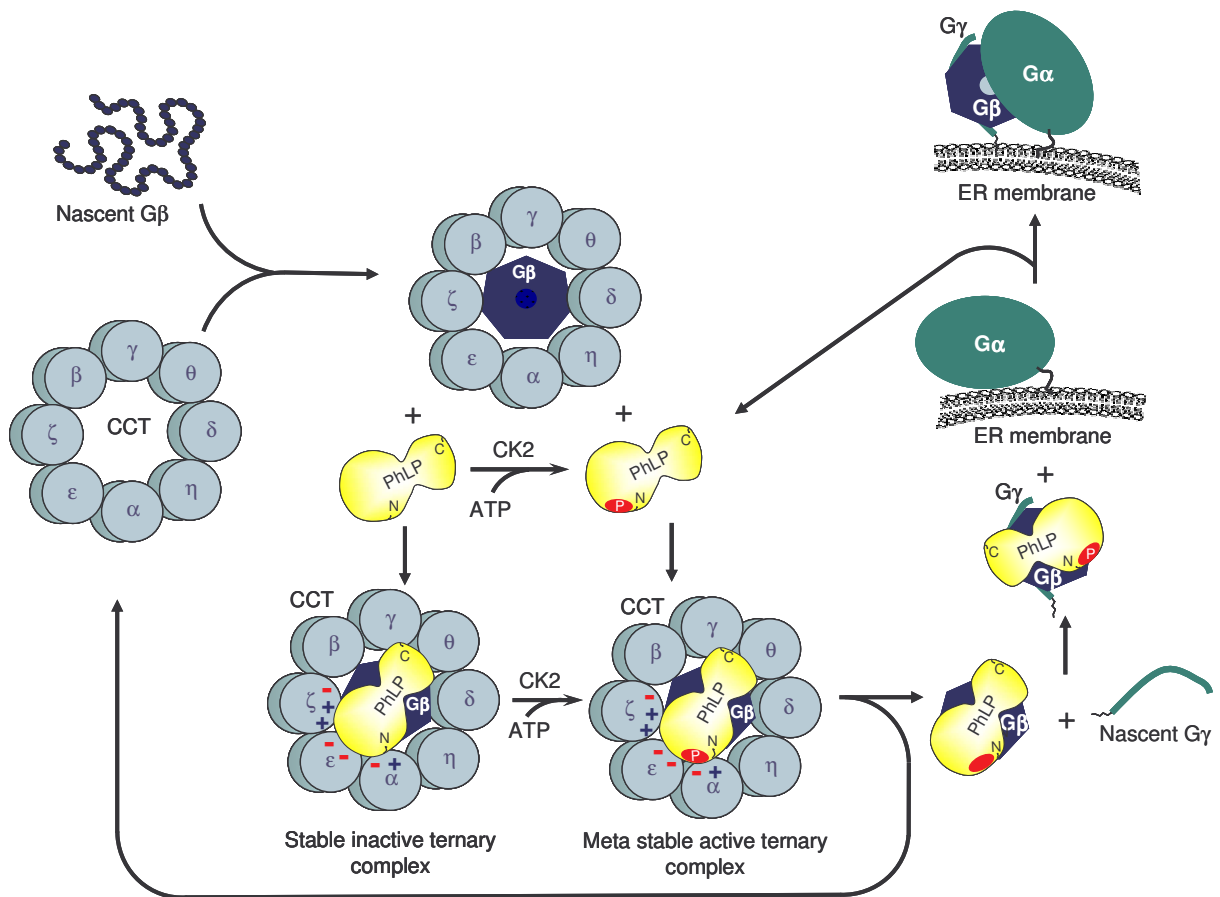
This result would not be expected in the first mechanism in which  $G\gamma$  binding would be required for release of  $G\beta$  from CCT. Similarly, the observed lack of increase in the  $G\beta$  dissociation rate upon co-expression of  $G\gamma$  with PhLP would be predicted by the second mechanism 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.** HEK-293 cells were transfected with Flag- $G\beta_1$ , HA- $G\gamma_2$ , or PhLP variants as indicated. Cells extracts were immunoprecipitated with an antibody to CCT $\epsilon$  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-G $\beta$  complex from CCT that can then associate with G $\gamma$  to form the G $\beta\gamma$  dimer. This conclusion is also supported by the previously reported observation that PhLP forms a stable complex with G $\beta$  that does not include G $\gamma$  (Lukov et al., 2005).



**Figure 3-7. CK2 phosphorylation-dependent release model of G $\beta\gamma$  assembly.** A model is proposed in which nascent G $\beta$  forms a ternary complex with CCT and PhLP. If PhLP is not phosphorylated, the ternary complex is stable and PhLP-G $\beta$  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 CCT $\alpha$  or  $\epsilon$  apical domains. Once released, the PhLP-G $\beta$  complex binds G $\gamma$ , forming the G $\beta\gamma$  dimer. The dimer then associates with G $\alpha$  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 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 (Lukov et al., 2005; Humrich et al., 2005). Phosphorylation of PhLP at S18-20 by CK2 was required for Gβγ 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 Gβγ assembly (Figure 3-7). There are five important steps in this mechanism: 1) the nascent Gβ polypeptide binds CCT. This is a stable complex that releases Gβ 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-Gβ and the Gβγ 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-Gβ dimer. 3) PhLP-Gβ dissociates from CCT. The structure of the Pdc-Gβγ complex shows that Pdc binds Gβ on the opposite face as Gγ (Gaudet et al., 2006), predicting that the Gγ binding site on Gβ would be free in the PhLP-Gβ dimer. 4) Gγ binds Gβ forming a PhLP-Gβγ complex. This complex is stable with a 100 nM binding affinity (Savage et al., 2000). However, both the Gα binding site and the membrane association surface of Gβγ overlap

extensively with the PhLP binding site (Savage et al., 2000).  $G\alpha$  and PhLP share similar binding affinities for  $G\beta\gamma$ . However, significantly more  $G\alpha$  is present in the cell, and there is an abundance of membrane surface on the endoplasmic reticulum and Golgi for  $G\beta\gamma$  binding. Therefore, in the cell PhLP would be expected to be released from  $G\beta\gamma$ . 5)  $G\beta\gamma$  associates with  $G\alpha$  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  $G\beta\gamma$  assembly.

This model readily explains the dominant negative effect of the PhLP S18-20A and PhLP  $\Delta$ 1-75 variants. These variants form PhLP- $G\beta$ -CCT ternary complexes that do not release PhLP- $G\beta$  for  $G\gamma$  binding. Such stable ternary complexes would also block the endogenous, phosphorylated PhLP from forming competent ternary complexes capable of releasing PhLP- $G\beta$  for  $G\gamma$  binding. Previous explanations of the dominant negative effect of PhLP S18-20A which postulated that unphosphorylated PhLP would block  $G\beta$  and  $G\gamma$  association with CCT (Humrich et al., 2005) or that unphosphorylated PhLP would form a PhLP- $G\beta$  complex that would not accept  $G\gamma$  (Lukov et al., 2005) are incomplete.

*Phosphorylation-induced conformational changes* – Although CK2 phosphorylation increases PhLP's binding to CCT in the absence of  $G\beta$  (Lukov et al., 2006), PhLP phosphorylation was necessary for the release of PhLP- $G\beta$  from CCT in the presence of  $G\beta$  (Figures. 3-4,5). The difference between these observations may stem from differences in the structures of the PhLP-CCT and PhLP- $G\beta$ -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<sub>18</sub>SSDEDES) 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 G $\beta\gamma$  assembly and its regulation by CK2 phosphorylation suggest that PhLP's interactions with G $\beta$  and CCT could be targeted by therapeutics to control the levels of G $\beta\gamma$  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).

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