2010-03-12

Role of Members of the Phosducin Gene Family in Protein Translation and Folding

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Role of Members of the Phosducin Gene

Family in Protein Translation

and Folding

Nana Kwasi Sono-Koree

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

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April 2010

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL
of a dissertation submitted by
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This dissertation 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 dissertation of Nana Kwasi Sono-Koree 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

Role of Members of the Phosducin Gene

Family in Protein Translation

and Folding

Nana Kwasi Sono-Koree

Department of Chemistry and Biochemistry

Doctor of Philosophy

G proteins regulate various physiological processes by way of transducing a wide variety of signals ranging from hormonal to sensory stimuli. Malfunctions in G protein signaling lead to numerous diseases. G protein signaling begins with binding of a ligand to a G protein-coupled receptor resulting in a conformational change that leads to the exchange of a GDP for a GTP on Gα. The GTP bound α subunit dissociates for its stable G$\beta\gamma$ dimer partner. Gα-GTP and G$\beta\gamma$ control the activity of effector enzymes and ion channels that ultimately orchestrate the cellular response to stimulus. Current reports have shown phosducin-like protein (PhLP1) as a co-chaperone with the chaperonin-containing tailless complex polypeptide-1 (CCT) in the assembly of G$\beta\gamma$ dimer. However, the studies did not address the role of PhLP1 and CCT in the translation and eventual assembly of G$\beta\gamma$ dimer. The data presented in Chapter 2 shows a co-translational assembly of G$\beta\gamma$ dimer regulated by PhLP1 and CCT.

Chapter 3 discusses the role of PhLP2A and PhLP3 in CCT-mediated assembly of actin and tubulin in mammalian cells. PhLP2 and PhLP3 are members of the phosducin gene family that interact with CCT. Several studies in yeast suggest that PhLP2 promotes CCT-dependent β-actin folding while PhLP3 enhances β-tubulin folding. However, human PhLP2 has been shown to inhibit β-actin folding, indicating that PhLP2 and possibly PhLP3 have very different functions in humans than they do in yeast. As a result, this study investigates in depth the role of PhLP2 and PhLP3 in CCT-dependent β-actin and β-tubulin folding in human cells.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

C-	Carboxy-terminus
CCT	Chaperonin containing tailless complex polypeptide 1
DRiP78	Dopamine receptor-interacting protein
DMEM	Dulbecco's Modified Eagle Medium
DTT	dithiothreitol
G protein	Heterotrimeric GTP binding protein
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
Gα	G-protein alpha subunit
Gβ	G-protein beta subunit
Gβγ	G-protein beta and gamma subunit dimer
Gγ	G-protein gamma subunit
HA	Hemagglutinin
HEK-293T cells	Human embryonic kidney 293 cells
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
N-	Amino-terminus
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pdc	Phosducin
PhLP1	Phosducin-like protein 1
PhLP 2A	Phosducin-like protein 2A
PhLP 2B	Phosducin-like protein 2B
PhLP 3	Phosducin-like protein 3
PMSF	phenylmethylsulfonyl fluoride
siRNA	Short interfering RNA

CHAPTER 1

Protein Translation and Regulation

The human genome is made up of about 25,000 genes (Pennisi, 2003), each coding for a specific protein. The genetic information encoded is transcribed into mRNA which is subsequently translated into protein. The fact that proteins are the building blocks of life makes their synthesis central to cellular function, hence the need for proper control of protein translation and subsequent folding into their native three dimensional conformations.

Translation is the process by which genetic information contained in a messenger RNA (mRNA) is decoded and converted into a protein. The process takes place on large ribonucleoprotein complexes called ribosomes (Muckenthaler and Preiss, 2006). Translation is divided into three steps namely: initiation, elongation and termination. Translation initiation in eukaryotes begins with the formation of a pre-initiation complex which includes the 40S ribosomal complex and its associated factors at the 5' end of the messenger RNA (mRNA). The pre-initiation complex scans the mRNA until the AUG start codon is reached. The 60S ribosomal subunit joins the complex to form an elongation competent 80S ribosome. The process of translation initiation is shown in Figure 1-1 on the following page.

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The next step in the process is the elongation step which begins with an initial aminoacylated tRNA at the peptidyl P-site of the ribosome. An activated tRNA (aminoacylated tRNA) with the appropriate codon-anticodon pair binds to the A-site of the 80S ribosome. A peptide bond is formed between the previous amino acid and the newly arrived one. The resulting peptide is translocated from the A-site to the P-site leaving the A-site free for another activated tRNA to start another elongation cycle.
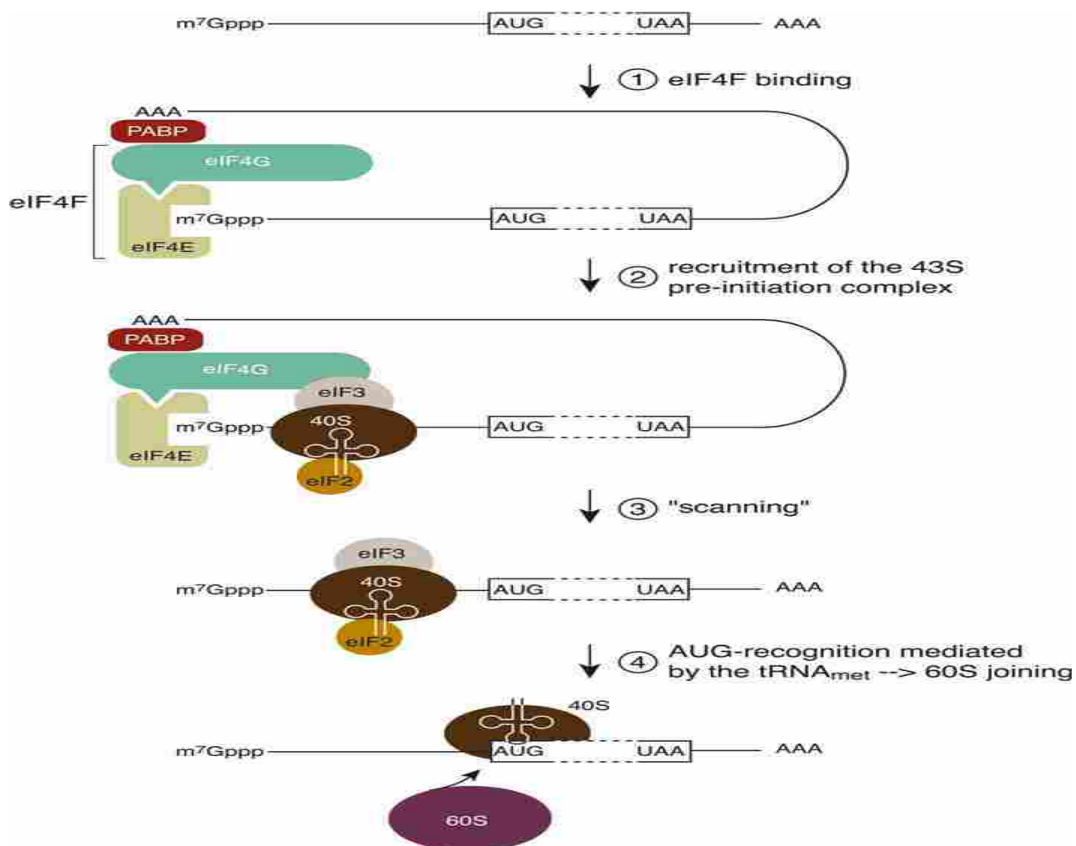
Figure 1-1. Initiation of Translation in Eukaryotes
Depicted is a typical eukaryotic mRNA with the post-transcriptional end modifications, a 5' cap structure and a 3' poly (A) tail. The protein-coding region is marked by start and stop codons. First, the eIF4F complex consisting of eIF4E, eIF4G, and eIF4A binds to the cap structure. The interaction between PABP and eIF4G leads to a pseudo-circularisation of the mRNA. The small ribosomal subunit (40S) is then recruited to the mRNA together with the initiation factors eIF3, eIF2 and the initiator-tRNAmet. This so-called 43S-preinitiation complex then moves along the mRNA in a process termed 'scanning'. The codon/anticodon interaction identifies the AUG start codon. This leads to the release of initiation factors and joining of the large (60S) ribosomal subunit. The formation of the complete 80S ribosome completes the initiation process and polypeptide synthesis as directed by the open reading frame can begin (Muckenthaler and Preiss, 2006).

The final step in the process of polypeptide synthesis is the termination step

which occurs when a stop codon reaches the A-site of the 80S ribosome. Through the

assistance of a number of eukaryotic release factors (eRF1 & 3), the resulting

polypeptide is released from the ribosome (Muckenthaler and Preiss, 2006). The 80S

ribosome subsequently dissociates into its 40S and 60S as shown in Figure 1-2.
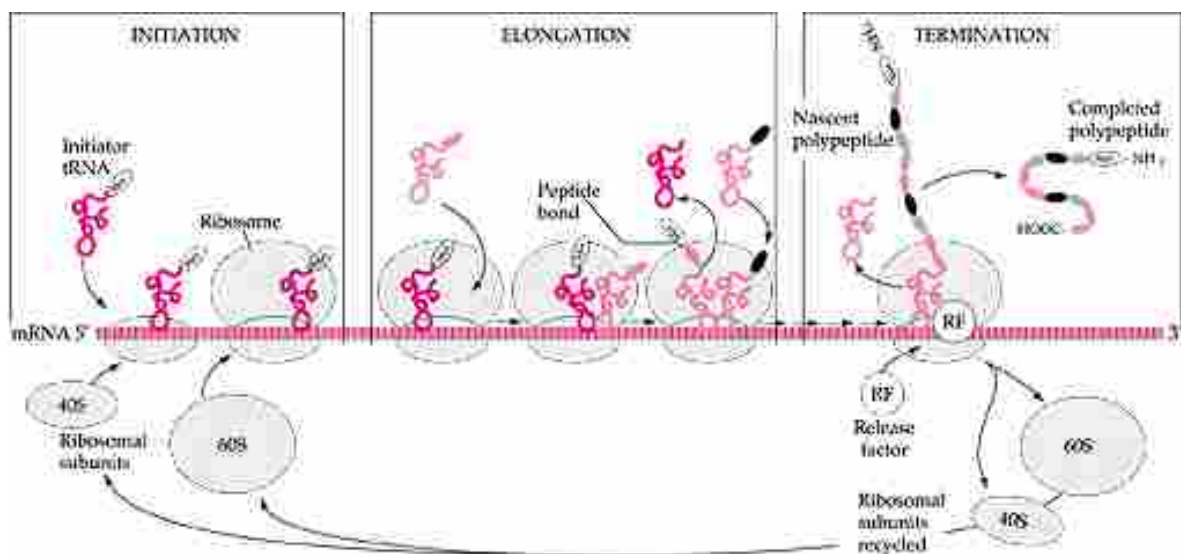
Figure 1-2. Schematic representation of the events of eukaryotic translation.
The initiation steps bring together the 40S and 60S ribosomal subunits, mRNA, and the initiator tRNA, which
is complexed to the amino acid methionine (Met). During elongation, amino acids are brought to the
polysome, and peptide bonds are formed between the amino acids. The sequence of amino acids in the
growing protein is directed by the sequence of nucleic acid codons in the mRNA. After the last peptide bond
of the protein has been made, one of the codons UAG, UGA, or UAA signals the termination of translation.
The ribosomal subunits and message can be reutilized (Gilbert, 2006).

Despite the fact that each of the three phases of translation can be under the

influence of a number of physiological and pathological processes, the initiation step

is the main target of a number of control mechanisms. Many biological processes

including cellular growth, embryonic development, and response to environmental

and biological cues rely heavily on translational control. Defects in translation or its control can lead to a number of diseases such as cancer, tissue hypertrophy and neurodegeneration (Proud, 2007). Translational control is one of the many means used by cells to ensure a steady internal environment.

Control of translation could be global where most or all cellular mRNAs are equally affected, or selective where specific mRNAs are affected (Sonenberg et al., 2000). The activity of most translation initiation factors is essential to the regulation of mRNA translation. The activity of these initiation factors depends on their phosphorylation status which is regulated by kinases and phoshatases. These kinases and phosphatases are regulated by two major signaling pathways namely: the Ras/MAPK and the PI3-K/Akt/mTOR pathways. The Ras/MAPK pathway regulates the phosphorylation of cap binding protein eIF4E while the PI3-K/Akt/mTOR pathway regulates the phosphorylation of ribosomal protein S6, eIF4G and 4E-binding protein (Muckenthaler and Preiss, 2006) . The phosphorylation status of these translation initiation factors can either stimulate or repress translation. This is seen in disease states such as cardiac hypertrophy which is characterized by inappropriate stimulation of protein accumulation due to signal-induced phosphorylation of translation factors and also under cellular stress conditions such as increase in temperature or the lack of glucose where a general inhibition of translation occurs.

The translation of specific mRNAs can also be controlled by interactions between secondary structures within the 5' UTR or 3' UTR and regulatory proteins. An example of this is seen with the iron absorbing protein ferritin whose expression is regulated by the level of intracellular iron. When the level of iron in the cell is low,

the iron regulatory proteins 1 or 2 (IRP-1 or IRP-2) bind to a secondary structure within the 5' UTR region of the ferritin mRNA called the iron responsive element (IRE). This RNA-protein interaction blocks translation initiation. On the other hand, if the level of iron in the cell increases, the IRP proteins dissociate and translation of ferritin is restored (Sonenberg et al., 2000). Another example of translational control as a result of RNA-protein interaction is seen with 15-lipoxygenase (LOX). The 3' UTR region contains a differentiation control element (DICE) that mediates translation repression by binding with heteronuclear ribonuclear proteins (hnRNP) K and E1. The RNA-protein interaction inhibits translation at the 60S subunit-joining step (Muckenthaler and Preiss, 2006). Organisms are in constant interaction with a rapidly changing environment which necessitates the use of regulatory tools. Regulation of protein translation and folding is nature's way of preserving homeostasis so as to avoid disease states.

Protein Folding

A unique characteristic of most living systems is the ability of its component molecules to assemble with precision. The ability of proteins to fold into their three dimensional structure and form functional complexes with other proteins is an example of such assembly. The fact that every protein in a cell has its unique functions and can only perform such functions if it is correctly folded into its native structure makes protein folding a key element of cellular function, and defects in the folding process can lead to serious pathological conditions.

Proteins are synthesized on ribosomes where genetic information transcribed into a messenger RNA is translated into polypeptides. Protein folding can be co-translational where it is initiated before the completion of synthesis in which the nascent polypeptide is still attached to the ribosome (Hardesty and Kramer, 2001) or after release from the ribosome where they are either folded in the cytoplasm or in specialized compartments like the mitochondria or the endoplasmic reticulum (Bukau and Horwich, 1998). Failure of proteins to fold correctly or to remain folded leads to malfunctioning of living systems and disease states such as cystic fibrosis, Alzheimer's and Huntington's disease (Thomas et al., 1995). To ensure that proteins remain in their three dimensional native states, certain macromolecules called molecular chaperones are present in all types of cells and cellular compartments. These molecules ensure that the process of protein folding takes place efficiently by reducing the probability of competing reactions (Dobson, 2003).

One of the unique ways adopted by nature to avoid diseases is the degradation of misfolded proteins. In eukaryotes, since most of the proteins synthesized are excreted to the extracellular environment which lacks molecular chaperones, it is important that only correctly folded proteins are exported. These secreted proteins are translocated into the endoplasmic reticulum where they are correctly folded before export by a complex network of chaperones. These proteins undergo a quality–control check before they are finally exported as shown in Figure 1-3 on the following page (Hammond and Helenius, 1995). Misfolded proteins can form aggregates within cells or in the extracellular space leading to pathological conditions such as Alzheimer's and Parkinson's diseases (Dobson, 2001). There are several signaling pathways in

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cells that are regulated by specific proteins. Misfolding or translation inhibition of

such proteins leads to inhibition of the signaling pathway and ultimately the specific

physiological response. One such signaling pathway is mediated by G proteins and is

the topic of this thesis.
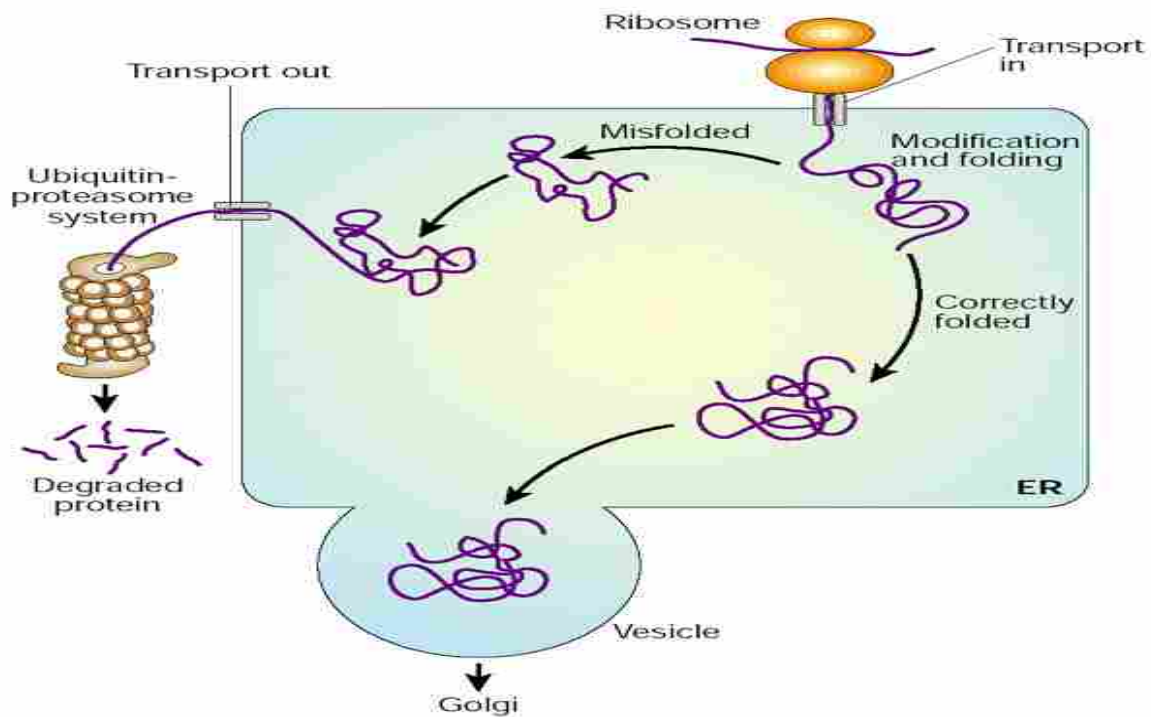
Figure 1-3. **Regulation of protein folding in the ER.**
Many newly synthesized proteins are translocated into the ER, where they fold into their three-dimensional structures with the help of a series of molecular chaperones and folding catalysts (not shown). Correctly folded proteins are then transported to the Golgi complex and then delivered to the extracellular environment. However, incorrectly folded proteins are detected by a quality-control mechanism and sent along another pathway (the unfolded protein response) in which they are ubiquitinated and then degraded in the cytoplasm by proteasomes (Dobson, 2003)

An intriguing class of oligomeric, high-molecular-weight chaperones with the

unique ability to fold some cytosolic proteins that cannot be folded by simpler

chaperone systems are called chaperonins (Frydman, 2001). This unique group of

chaperones consists of two-ring assemblies with a central cavity where substrate

polypeptides bind to reach their native state (Bukau and Horwich, 1998; Gutsche et

al., 1999). Prokaryotes utilize the class I chaperonin GroEL, whose ring assembly is

made up of seven identical subunits and requires a co-chaperone GroES, which caps

the central cavity creating a protected space for the substrate polypeptide to fold

(Horwich et al., 2009). Eukaryotes employ a class II chaperonin, CCT (chaperone-

containing TCP1, also called TRiC, TCP-1 ring complex) (Bukau and Horwich, 1998;

Gutsche et al., 1999), which is a hetero-oligomeric complex with eight unique yet

homologous subunits per ring (Valpuesta et al., 2002). Each subunit consist of three

domains with specific functions: an equatorial ATP-binding domain, an apical

domain that is involved in substrate binding, and a central hinge domain that enables

communication between the equatorial domain and the apical domain (Spiess et al.,

2004). CCT was initially proposed to fold only actin and tubulin, but many other

substrates have been discovered (Thulasiraman et al., 1999), including a class of

proteins containing WD 40 repeats, a 40 amino acid repeat ending in a tryptophan-

aspartic acid (WD) sequence. WD40 repeats with β-propeller structures have been

identified through proteomic studies to interact with CCT (Gavin et al., 2002; Ho et

al., 2002). Many CCT substrates cannot be folded by other prokaryotic and

eukaryotic chaperones (Tian et al., 1995), making CCT essential for the folding of

proteins that regulate important physiological functions. Many of the CCT substrates

identified so far are subunits of either a homo- or hetero-oligomeric complex (Spiess

et al., 2004), and their folding and function is coupled to their incorporation into their

higher-order assemblies (Dunn et al., 2001). CCT can bind co-translationally to nascent polypeptides as they emerge from ribosomes (Frydman et al., 1994; McCallum et al., 2000; Melville et al., 2003; Siegers et al., 2003). Binding to CCT requires the assistance of upstream chaperones (Frydman, 2001) and just like GroEL, CCT recognizes its substrates using its apical domain, but the exact location of the binding sites within this domain is not defined (Frydman, 2001). As a result of the sequence diversity in apical domains, an attractive hypothesis is that different subunits recognize different types of motifs, including both polar and hydrophobic-recognition sites (Frydman et al., 1994). The apical domains of CCT are in an open conformation that exposes the substrate-binding sites in the absence of ATP (Frydman et al., 1992; Gao et al., 1992; Llorca et al., 1999b). Addition of ATP induces formation of the closed lid, which confines the substrate in the central cavity (Spiess et al., 2004). After release of the γ-phosphate, ADP-bound CCT reverts to its open state (Melki and Cowan, 1994). Kinetic studies suggest that subunits of one ring bind to ATP in a positively cooperative manner (Kafri and Horovitz, 2003; Kafri et al., 2001) an indication of a concerted mode of action during lid closure. The fact that CCT is linked to several pathological states such as sensory neuropathy (Lee et al., 2003) and tumor causing mutations in the CCT binding sites of some of its substrates such as the VHL tumor suppressor (Spiess et al., 2004) makes it a key element in the regulation of cellular functions and subsequent physiological processes. An important CCT substrate is the β subunit of the heterotrimeric G protein (Gβ) (Lukov et al., 2005). Gβ is a WD40 repeat protein that forms a seven-bladed β-propeller structure (Sondek et al., 1996; Wall et al., 1995) and plays a key

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role in G protein mediated signal transduction. To understand the physiological

importance of Gβ and its CCT-dependent folding, a brief overview of G protein

signaling is required.
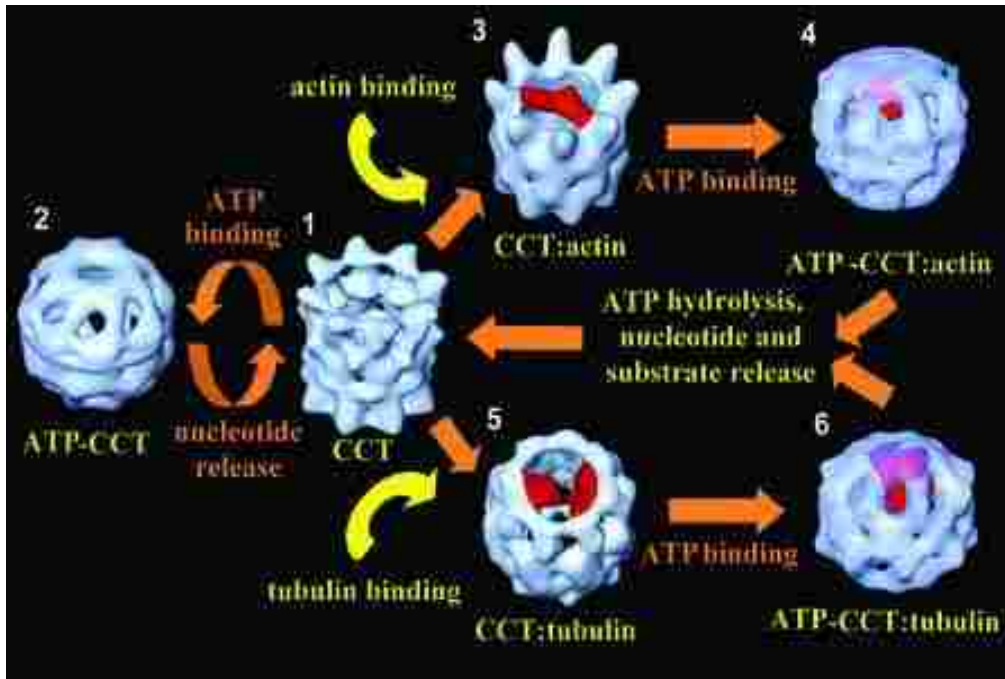
Figure 1-4. Model of the structural changes undergone by CCT during its functional cycle.
The nucleotide-free, substrate-free structure (1) shows an open conformation of its apical domains,
which undergoes large structural changes upon ATP binding such that the cavity is closed (2). The
substrate-free structure is able to bind unfolded actin (3) and tubulin (5) molecules in a quasi-native
conformation. Binding of tubulin generates a more closed conformation of the apical domains than
observed without substrate or after actin binding. ATP binding to the CCT–α-actin (4) or CCT–β-
tubulin (6) complexes induces conformational changes of the chaperonin apical domains that seal
the cavity using their helical extensions. The more downward and inward distribution of the apical
domains in the CCT–β-tubulin complex compared with the CCT–α-actin complex is maintained
after ATP binding (Llorca et al., 2001).

G Protein Signal Transduction and its Regulation

Most extracellular signals such as hormones, neurotransmitters and sensory stimuli are relayed into cells through activated plasma membrane bound receptors. G protein-coupled receptors (GPCRs) form the largest class of such receptors with greater than 1% of the human genome dedicated to their synthesis (Takeda et al., 2002). GPCRs have a membrane-spanning region comprised of seven helices that activates G proteins upon ligand binding, leading to the transduction of signals to several intracellular signaling pathways. These activated signals interact with each other forming a network that regulates many components of the cell's machinery such as metabolic enzymes, ion channels, transporters and transcriptional regulators (Neves et al., 2002). These cellular activities in turn regulate many systemic functions such as gonadal development, learning and memory and general organismal homeostasis. The physiological importance of GPCRs has been demonstrated with knockout models showing pathological phenotypes related to the nervous, endocrine, sensory and cardiovascular systems (Yang et al., 2002). Due to their physiological importance, GPCRs are a major therapeutic target for most pharmaceutical companies with annual sales of several billion dollars (Overington et al., 2006).

Heterotrimeric G proteins are guanine nucleotide binding proteins that interact with the intracellular domain of GPCRs and connect receptors with effectors. In so doing, they transduce extracellular signals from hormones, neurotransmitters, chemokines and paracrines to intracellular effectors. G proteins consist of three different subunits namely Gα, Gβ and Gγ. Currently 16Gα, 5Gβ and 12Gγ human genes have been identified with the existence of several splice variants (Gudermann,

2006). Based on sequence homology of the Gα subunits, G proteins have been classified into four families namely Gα_s, G$\alpha_{q/11}$, G$\alpha_{i/o}$ and G$\alpha_{12/13}$. G protein α subunits have a Ras-like GTPase domain which is used for GTP hydrolysis and a helical domain which buries the bound nucleotide in the protein core (Gudermann, 2006). Gαs have the unique characteristic of being the only G protein subunit that binds guanine nucleotides and that has the ability to hydrolyze bound GTP.

Among the Gβ subunits, all but Gβ_5 share between 78-80% sequence homology (Schwindinger and Robishaw, 2001). Gβ_5 shares about 55% sequence homology with the others and contains an additional 13 amino acid residues at the N-terminus. It is only expressed in the retina and central nervous system (Watson et al., 1994). As mentioned previously, Gβ subunits are made up of seven WD40 repeating sequence motifs that form distinct β sheets that make a seven bladed propeller structure which is connected by a loop to a 25 residue N-terminal α-helix (Lambright et al., 1996; Sondek et al., 1996).

Gγ subunits are more structurally diverse compared to Gβ. They share between 27-76% sequence homology and all undergo posttranslational modification of a carboxyl terminal cysteine residue in a conserved CaaX motif by an isoprenyl group. This modification is essential for association of the G$\beta\gamma$ heterodimer with membranes. Gγ binds to Gβ in an extended conformation devoid of intrachain tertiary interactions (Gudermann, 2006). Even though G$\beta\gamma$ is a stable heterodimer, not all combinations of Gβ and Gγ subtypes can form dimers (Mende et al., 1995).

Family	α_s	α_i	α_q	$\alpha_{12/13}$	βγ
Members	Gαs	Gαi1/2/3	Gαq, Gα14	Gα12	β (1–5)
	Gαsxl	Gαo-Gαt	Gα11, Gα15/16	Gα13	γ (1–8)
	Gαolf	Gαz, Gαgust			
Effectors	Adenylyl cyclases	Adenylyl cyclases	PLCβ	Rho GEFs	Ion channels
	Increased cAMP	Inhibition of cAMP	DAG	Rho	PI3Kγ
	PKA	Ion channels	Ca²⁺		PLCβ
		Phosphodiesterases	PKC		Adenylyl cyclases
		Phospholipases			
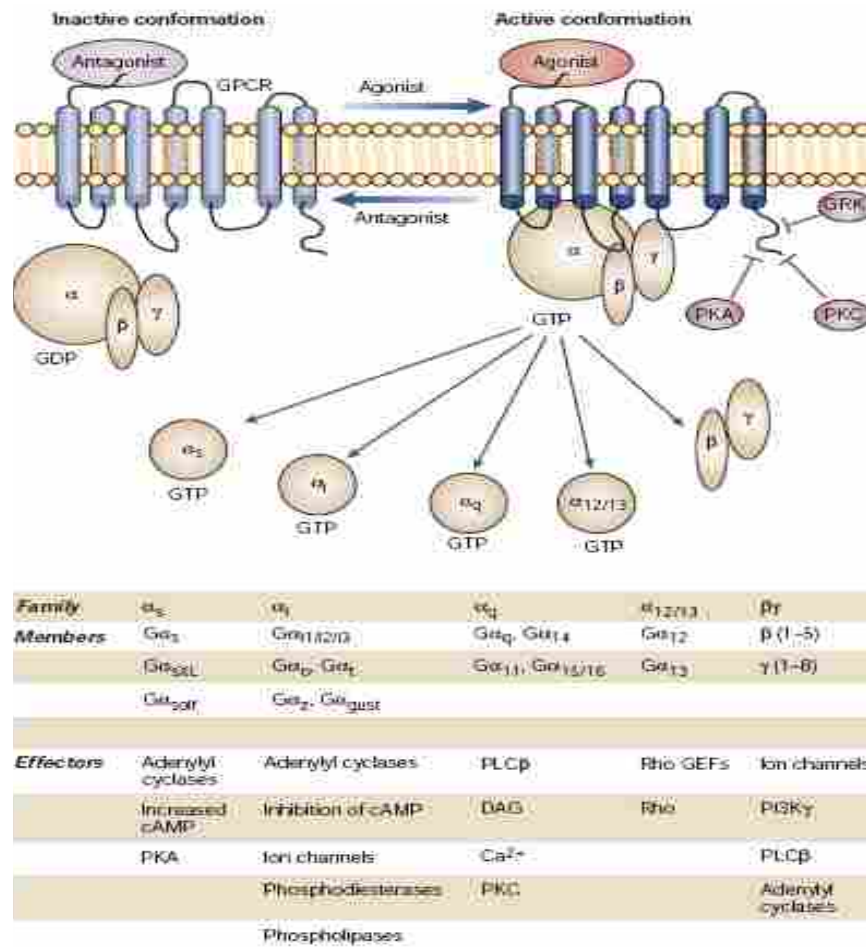
Figure 1-5. G proteins and Second Messengers.
When activated by agonists, G-protein-coupled receptors (GPCRs) profoundly change the conformation of their transmembrane α-helices, which uncovers previously masked G protein binding sites. This, in turn, promotes GDP-GTP exchange on the α-subunit, which results in their activation. Consequently, Gβγ and GTP-bound Gα proteins stimulate effector molecules. The biochemical changes that are induced are highly dependent on the individual receptor-coupling specificity for each of the four families of mammalian G-protein α-subunits: α_s, α_i, α_q or $\alpha_{12/13}$ (see Figure). Receptors that are coupled to Gα$_s$ activate adenylyl cyclases, thereby increasing cyclic-AMP levels; whereas those that stimulate the Gα$_i$-family members inhibit adenylyl cyclases and reduce cAMP levels, as well as activating phospholipases and phosphodiesterases. Receptors that are coupled to the Gα$_q$ family promote the activation of phosphatidylinositol-specific phospholipases, such as phospholipase C-β (PLCβ), which hydrolyses phosphatidylinositol-4, 5-bisphosphate to generate inositol triphosphate (IP$_3$) and diacylglycerol (DAG). These elevate intracellular calcium concentrations and induce the activation of several protein kinases, including protein kinase C (PKC) (Sodhi et al., 2004).

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It is not clear what determines selectivity even though the N terminus of Gβ is important for dimerization (Lupas et al., 1992). Gβ1-4 can form irreversible dimers with most Gγ subunits (Dingus et al., 2005). In contrast, Gβ5 only forms dimers with RGS proteins of the R7 family (Witherow and Slepak, 2003).

G protein signaling is initiated when a ligand (agonist) binds to the extracellular domain of a GPCR resulting in the exchange of a GDP for a GTP on the inactive Gα subunit bound to G protein βγ dimer. The binding of GTP to Gα induces conformational changes in three flexible switch regions resulting in its dissociation from Gβγ dimer. Both the activated Gα-GTP and the Gβγ dimer relay signals to downstream effectors including ion channels, adenylyl cyclase, phosphodiesterases and phospholipases which give rise to changes in several second messengers that regulate many physiological processes (Offermanns, 2003). Deactivation of G protein signaling is caused by the intrinsic hydrolysis of GTP to GDP by Gα, a process that is enhanced by members of the Regulators of G protein signaling (RGS) super family (Koelle, 1997). Figure 1-5 is a depiction of G protein signal transduction when a GPCR is either activated or deactivated.

G protein signaling is highly regulated either by controlling the number and activity of GPCRs on the cell surface or through the hydrolysis of GTP by members of the RGS family of proteins. The number and activity of GPCRs are controlled by downstream effector kinases such as protein kinase A or C or by G protein receptor kinases (GRK) (Gainetdinov et al., 2004). Phosphorylation by these kinases leads to

receptor deactivation. In the case of GRK, phosphorylation leads to the binding of arrestin, which in turn recruits a clathrin adaptor protein (AP2) leading to the inclusion of the GPCR into a clathrin-coated pit and final internalization into endocytotic vesicles (Luttrell and Lefkowitz, 2002). The resulting vesicles are either sent to the lysosomes where they are degraded, or they are recycled back to the cell membrane.

Regulation of G protein signaling has also been proposed by Gβγ dimer binding partners such as phosducin and PhLP1 that obstruct the dimer from activating downstream effectors as a result of their interaction. However, more recent evidence suggests that members of the phosducin family are not inhibitors of Gβγ signaling but rather they are essential co-chaperones with the cytosolic chaperonin complex (CCT) to fold and assemble Gβγ dimers (Willardson and Howlett, 2007).

The Phosducin Family

The phosducin family consist of three subgroups namely phosducin-I, phosducin-II and phosducin-III based on phylogenetic analysis of 33 protein sequences from mammals, invertebrates, plants and eukaryotes (Blaauw et al., 2003). Members of each subgroup share extensive sequence homology at their C-terminal thioredoxin-like domains while the N-terminal domains are very divergent. Figure 1-6 shows the phosducin family tree. PhLP1 is a 34kDa member of the phosducin gene family that was initially discovered as an ethanol-induced gene in cultured neurons (Miles et al., 1993). It shares 65% sequence homology with phosducin, and the two constitute subgroup I of this gene family (Miles et al., 1993). PhLP1 is

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expressed significantly in most tissues (Schroder and Lohse, 2000) and was initially

thought to inhibit G protein signaling by sequestering Gβγ and blocking its

interaction with effectors and Gα (Thibault et al., 1997). This sequestration

hypothesis for phosducin and PhLP1 persisted for many years, but was brought into question by several inconsistencies, beginning with the low expression level of PhLP1 compared to Gβγ. In order to block Gβγ signaling in the cell, PhLP1 had to be over-expressed to well above the endogenous level (McLaughlin et al., 2002a). In addition, the deletion of the *phlp1* gene in chestnut blight fungus *Cryphonectria parasitica* (Kasahara et al., 2000) and *D. discoideum* (Blaauw et al., 2003)

Figure 1-6. The Phosducin Family Tree.
The phosducin family consists of three defined subgroups. The protein sequences of 33 phosducin homologues were obtained from different organisms and aligned. The alignment was used as input for the Phylip program to construct the phylogenetic tree. The numbers indicate bootstrap values (Blaauw et al., 2003).

resulted in a severe loss of G protein signaling, yielding the same phenotype as a Gβ

gene deletion. This result was the opposite of what would be expected if PhLP1

down regulated G protein signaling. These inconsistencies led to further studies in

which PhLP1 was found to be an essential chaperone of Gβγ dimer assembly instead

of an inhibitor of Gβγ signaling as initially proposed (Lukov et al., 2005).

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The first evidence of the chaperone function of PhLP1 was the discovery of a high affinity interaction between PhLP1 and CCT (McLaughlin et al., 2002a). Importantly, PhLP1 was shown to be an interacting partner instead of a folding substrate for CCT (McLaughlin et al., 2002a). Through cryo-electron microscopy, PhLP1 was shown to bind to the apical domain of CCT subunits above the folding cavity in a manner similar to the CCT co-chaperone prefoldin (Martin-Benito et al., 2004). Several subsequent studies showed that PhLP1 and CCT acted as co-chaperones in the assembly of the Gβγ dimer. First siRNA–mediated deletion of PhLP1 in mammalian cells significantly decreased Gβ1 expression and subsequent G protein signaling with no effect on Gβ1 mRNA levels (Lukov et al., 2005). Second, pulse chase experiments measuring the rate of assembly of Gβ1γ2 showed a 5-fold decrease when the cells were depleted of 90% of their PhLP1 and a 4-fold increase when PhLP1 was over-expressed (Lukov et al., 2005). A similar observation was seen in Dictyostelium where PhLP1 deletion resulted in cells completely devoid of Gβγ dimers (Knol et al., 2005). In addition, *in vitro* studies showed that nascent Gβ interacted with CCT (Wells et al., 2006). Together, these studies suggest that PhLP1 and CCT work together as partners to fold and assemble Gβ with its heterodimer partner Gγ.

The proposed mechanism by which PhLP1 and CCT assist in Gβγ assembly is shown in Figure 1-7. It begins with nascent Gβ binding to CCT, followed by PhLP1 binding to form a ternary complex. This ternary complex is stable unless PhLP1 is phosphorylated within serines 18-20 (Lukov et al., 2006; Lukov et al., 2005). Upon

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phosphorylation, a PhLP1-Gβ complex dissociates from CCT and subsequently associates with Gγ, forming a PhLP1-Gβγ complex with a 100 nM binding affinity (Savage et al., 2000). It is believed that the resulting dissociation is due to electrostatic repulsion between the negatively charged phosphate moiety and negative charges on the CCT apical domains. In this manner, CCT and PhLP1 enhance G protein signaling by helping to assemble the Gβγ heterodimer, which plays an essential role in the interaction of Gα with receptors and in the regulation of many downstream effectors.
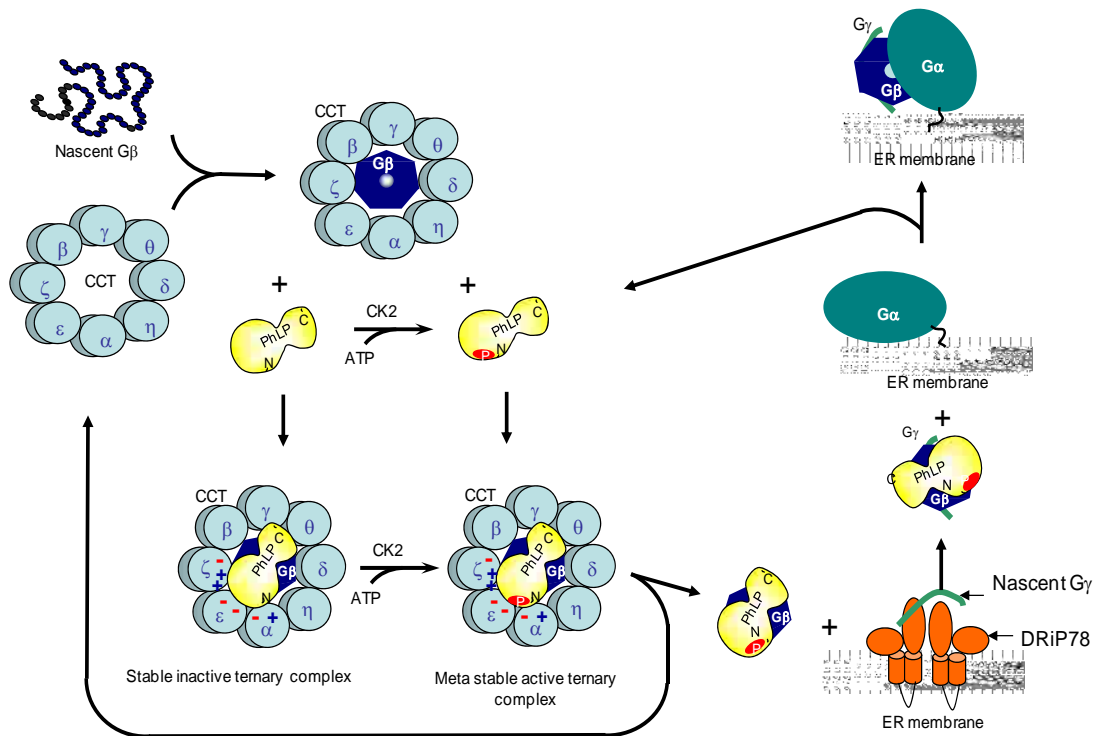

Figure 1-7. Phosphorylation-dependent assembly of the Gβγ heterodimer by CCT and PhLP1
According to the above model, nascent Gβ initially binds to CCT, followed by PhLP1 binding. If PhLP1 is phosphorylated at positions S18-20, depicted by a red oval, PhLP1-Gβ is released and associates with Gγ to form the heterodimer.

In addition to its role as a co chaperone for the assembly of Gβγ heterodimer, PhLP1 has been shown through bioluminescent energy transfer (BRET) studies to interact with an endoplasmic reticulum-bound protein called DRiP78 which has been proposed to stabilze Gγ until it finds its stable interacting partner Gβ (Dupre et al., 2007a).

DRiP78 in G protein Signaling

DRiP78 is an endoplasmic reticulum-bound protein known for its transport of seven transmembrane receptors with an FXXXFXXXF motif in their C-terminal tail to the plasma membrane (Dupre et al., 2007a). DRiP78 contains two centrally located transmembrane domains with cytosolic orientation of both its N and C termini (Bermak and Zhou, 2001) and a 70 amino acid J domain which is near the N terminus on the cytosolic surface of the endoplasmic reticulum (Qiu et al., 2006). It is proposed that DRiP78 initially co-localizes with Gγ before Gβγ dimer formation (Dupre et al., 2007a). In the presence of Gβ, Gγ no longer interacts with DRiP78, suggesting that DRiP78 releases Gγ for interaction with Gβ (Dupre et al., 2007a). Moreover, the binding of PhLP1 to DRiP78 suggests that PhLP1 may bring the Gβ subunit to DRiP78 where it can bind Gγ and form the Gβγ dimer (Dupre et al., 2007a). Interestingly, the assembly of Gβγ dimers is reduced when DRiP78 level is reduced using shRNA in HEK293 cells (Dupre et al., 2007a) an affirmation of the above suggestion. There appears to be some specificity in DRiP78 interactions with Gγ subunits, preferring $G\gamma_2$ and $G\gamma_3$ which are both in Gγ subfamily II and are very homologous at the sequence level (Dupre et al., 2007a)

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PhLP2- A CCT-dependent protein folding interacting partner

PhLP2 is one of the Phosducin-like genes found in the genomes of many eukaryotes including mouse, humans, yeast, zebra fish and fly. Deletion of PhLP2 in yeast is lethal as spore products that formed failed to grow (Flanary et al., 2000). In *D. discoideum*, PhLP2 disruption led to a decrease in growth rate and subsequent collapse of cultured cells after 16-17 cell divisions (Blaauw et al., 2003). There are two PhLP2 genes in the genomes of mammals such as humans and mice. These two share about 57% sequence homology (over 239 residues) and are called PhLP2A and PhLP2B (Wilkinson et al., 2004). While PhLP2A is ubiquitously expressed, PhLP2B is only expressed in male and female germ cells undergoing meiotic maturation (Lopez et al., 2003). Through confocal microscopy studies, PhLP2A was shown to be localized in the cytoplasm (Wilkinson et al., 2004). Just like PhLP1, PhLP2 interacts with the cytosolic chaperone containing tailless complex (CCT) as a folding partner and not as a substrate (Stirling et al., 2007). PhLP2 has been suggested as a possible CCT co-chaperone in the folding of components essential for regulating cell cycle progression (Stirling et al., 2007), but the identity of these components has not be determined.

Studies of temperature-sensitive mutants of PhLP2 in yeast suggest a possible role of PhLP2 with CCT in the folding of cytoskeletal proteins actin and tubulin (Stirling et al., 2007). However, *in vitro* studies showed that PhLP2A formed an inactive ternary complex with CCT that inhibited actin folding (Stirling et al., 2007). In contrast, subsequent *in vitro* studies showed that yeast PhLP2 enhanced actin folding by CCT by seven fold over the basal level (McCormack et al., 2009). Thus,

most of the evidence indicates that PhLP2 is a co-chaperone for actin folding in yeast.

PhLP2A could also be involved in the folding of proapoptotic factors due to complete

inhibition of caspase-3 processing following the initiation of Bax-induced

programmed cell death in PhLP2A siRNA knockdown cells (Wilkinson et al., 2004).

PhLP3 - A CCT interacting partner involved in the folding of β-tubulin and other CCT substrates

PhLP3, which is also called APACD or TXNDC9 in mammals, interacts with

CCT just like PhLP1 and PhLP2 (Stirling et al., 2006). Cryo-EM studies have shown

that PhLP3 and CCT form a complex (Stirling et al., 2006). PhLP3 has been linked

to G protein signaling in yeast (Flanary et al., 2000), but has also been implicated in

tubulin function in yeast and *C. elegans* (Stirling et al., 2006). Unlike PhLP2, PhLP3

deletion had no obvious phenotypic effect in *D. discoideum* (Blaauw et al., 2003). As

with PhLP2, there are discrepancies between the role of PhLP3 through *in vivo* and *in*

vitro studies. While *in vivo* genetic studies suggest PhLP3 as an enhancer of β-tubulin

folding (Lacefield and Solomon, 2003), *in vitro* studies suggest otherwise (Stirling et

al., 2006). Yeast studies of PhLP3 suggest it may be a down regulator for the

expression and folding of actin, a notion that is supported by *in vitro* studies (Stirling

et al., 2006). However the exact role of PhLP3 in actin folding is not clearly defined.

CHAPTER 2
CO-TRANSLATION ASSEMBLY OF THE G PROTEIN βγ DIMER

Summary

Current reports have shown phosducin-like protein (PhLP1) to be a co-chaperone with the cytosolic chaperonin complex CCT in the assembly of Gβγ dimer. However, the studies did not address the role of PhLP1 and CCT in the translation and eventual assembly of Gβγ dimer. This work outlines an elegant mechanism that links translation of the Gγ subunit to formation of the Gβγ dimer, bringing together the unstable Gβ and Gγ polypeptide chains in a way that avoids aggregation or degradation of the Gγ subunit.

Introduction

G proteins function as molecular switches, relaying signaling information from G protein-coupled receptors (GPCR) at the cell's extracellular surface to effector enzymes and ion channels on the cytoplasmic side of the plasma membrane. The signaling pathway is initiated by the binding of a ligand to the extracellular domain of the receptor which induces a conformational change that opens up the cytoplasmic side of the seven transmembrane helical bundle found in all GPCRs. This conformational change exposes an interaction site for the C-terminus of the G protein α subunit (Gα) (Farrens et al., 1996; Kobilka and Deupi, 2007; Scheerer et al., 2008); and the resulting binding of the receptor to Gα causes a conformational change in Gα that drives exchange of GDP for GTP in its guanine nucleotide binding pocket (Oldham and Hamm, 2008). GTP binding results in a rearrangement of the

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interface between Gα and the Gβγ dimer of the G protein heterotrimer, releasing both Gα-GTP and Gβγ for interactions with effectors (Preininger and Hamm, 2004; Sprang, 1997). By controlling effector activity, G proteins orchestrate cellular responses via changes in the concentration of important second messengers such as cyclic nucleotides, Ca^{2+} and phosphatidyl inositol lipids (Arshavsky et al., 2002; Drin and Scarlata, 2007; Hanoune and Defer, 2001; Hawkins and Stephens, 2007), by regulating membrane potential through K^+ and Ca^{2+} channels (Tedford and Zamponi, 2006; Xie et al., 2007), or by activating actin cytoskeleton rearrangements through nucleotide exchange factors for Rho GTPases (Worzfeld et al., 2008). Through these signaling pathways, G proteins regulate many important physiological processes, the malfunction of which results in numerous diseases ranging from heart failure (Pleger et al., 2007) to metastatic cancer (Juneja and Casey, 2009)

In order to perform its essential function, the G protein heterotrimer must be assembled post-translationally from its individual components. This is not a trivial task given the instability of both the Gβ and Gγ subunits prior to formation of the Gβγ dimer. This instability is overcome by a network of molecular chaperones that escort both the nascent Gβ and Gγ polypeptides until they are brought together to form the stable Gβγ complex. Recently, considerable progress has been made toward understanding the mechanism of Gβγ assembly. The Gβ subunit is assisted in folding into its seven-bladed β-propeller structure by the cytosolic chaperonin containing tailless complex polypeptide 1 (CCT, also called TCP1 ring complex (TRiC)) (Lukov et al., 2006; Lukov et al., 2005; Martin-Benito et al., 2004; McLaughlin et al.,

2002a; Wells et al., 2006). CCT is a large oligomeric protein complex made up of two rings of eight distinct but homologous subunits (Valpuesta et al., 2002). The two rings stack against each other to form a cylindrical structure with a central cavity on both ends, into which nascent polypeptides and unfolded proteins associate (Llorca et al., 1999). Each CCT subunit has intrinsic ATPase activity and upon ATP binding, a conformational change causes the tips of the CCT subunits to close over the protein substrate, creating a protected space in which the protein can fold (Llorca et al., 2001). Upon ATP hydrolysis, the folding cavity opens up and the folded protein, which has lost contacts with CCT during the folding process, is released into the cytosol (Llorca et al., 2001). Proteins identified as CCT substrates now number in the hundreds (Dekker et al., 2008), with possibly 5-10% of all cytoplasmic proteins being folded by CCT (Yam et al., 2008). Among CCT folding substrates, there is an enrichment in proteins with complex β-sheet structures as well as in polypeptides that are part of oligomeric protein complexes (Yam et al., 2008). Thus, the Gβ subunit is a typical CCT folding substrate.

A unique aspect of CCT-dependent Gβ folding is the inability of Gβ to release from CCT on its own, necessitating the co-chaperone phosducin-like protein 1 (PhLP1) to release Gβ from CCT and mediate its assembly with Gγ (Lukov et al., 2006). In this process, PhLP1 is believed to form a ternary complex with Gβ and CCT, with Gβ in the folding cavity and PhLP1 positioned above the cavity, contacting both Gβ and the tips of the CCT subunits (Lukov et al., 2006; Martín-Benito et al., 2004). Once Gβ is folded, it is released from CCT, possibly as a

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PhLP1-Gβ intermediate (Lukov et al., 2006; Lukov et al., 2005). In this intermediate, the Gγ binding site on Gβ is completely accessible (Gaudet et al., 1996), allowing Gγ to associate with Gβ. Gγ itself is also believed to require chaperones. This small 70 amino acid polypeptide does not form a stable structure in the absence of Gβ, but has been shown to bind the J-domain containing chaperone DRiP78 prior to its assembly with Gβ (Dupre et al., 2007b). Interestingly, PhLP1 was also reported to interact with DRiP78 (Dupre et al., 2007b), suggesting that the PhLP1-Gβ complex may contact DRiP78 and remove Gγ, forming the PhLP1-Gβγ complex. This complex is stable with a K_d of ~ 100 nM (Savage et al., 2000), but PhLP1 can dissociate from Gβγ through competition with Gα for the same binding site (Gaudet et al., 1999; Yoshida et al., 1994). Once PhLP1 is released and the Gαβγ heterotrimer is formed, it can be trafficked to the plasma membrane to interact with GPCRs and perform its signaling function (Marrari et al., 2007).

Interestingly, in experiments where PhLP1 function was inhibited by siRNA-mediated depletion or by over-expression of a dominant negative PhLP1 variant, not only was the rate of Gβγ assembly severely inhibited, but the synthesis of both nascent Gβ and Gγ appeared to be decreased as well (Lukov et al., 2005). This decrease did not seem to be simply a result of rapid degradation of the undimerized Gβ or Gγ, but rather from a possible negative feedback mechanism in which the synthesis of Gβ and Gγ was down-regulated by the inability of the subunits to form dimers (Lukov et al., 2005). The current study was initiated to further investigate this observation. The results suggest that in the process of translation, the N-terminus of

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Gγ forms an initial coiled-coil interaction with Gβ in the CCT folding cavity which causes translation of the remaining Gγ to stall. PhLP1 relieves this inhibition by releasing Gβ from CCT, allowing Gγ to finish its translation and simultaneously form the Gβγ dimer. Thus, Gβγ assembly appears to occur by an elegant co-translational mechanism, obviating the need for a Gγ chaperone to bring the dimers together.

Experimental Procedures

Cell Culture - HEK293T cells were cultured in DMEM/F-12 (50/50 mix) growth media supplemented with L-glutamine, 10% fetal bovine serum and 15 mM HEPES (Hyclone Scientific). In order to maintain active growth, cells were subcultured regularly but not beyond 15 passages.

Preparation of cDNA constructs - The pcDNA3.1 vectors containing N-terminally Flag-tagged human Gβ1 and N-terminally HA-tagged Gγ2 were obtained from the UMR cDNA Resource Center (www.cdna.org). Wild type human PhLP1 and the PhLP1 Δ1-75 N-terminal truncation variant each with a 3′ c-myc and His$_6$ tag were constructed in pcDNA3.1/myc-His B vector using PCR as described (Carter et al., 2004; Lukov et al., 2005). The pcDNA3.1(+)-Flag-GST and pcDNA3.1(+)-Flag-β-actin constructs were created by PCR amplification of the GST and β-actin cDNAs (Open Biosystems) with the primers 5'- BamHI-Flag-GST , 5'-BamHI-Flag-β-actin, 3'-XhoI-GST and XhoI-β-actin, digestion with BamHI and XhoI, and ligation into pcDNA3.1(+). The pcDNA3.1(+)-HA-Gγ-Flag-GST fusion construct was created by PCR amplification of the HA-tagged Gγ gene from pcDNA3.1(+)-HA-Gγ vector with the primers 5′-NheI-Gγ and 3′-KpnI-Gγ,digestion with NheI and KpnI, and ligation

into pcDNA3.1(+)-Flag-GST. The pcDNA3.1(+)-HA-Gγ-10-Flag-GST,

pcDNA3.1(+)-HA-Gγ-20-Flag-GST, and pcDNA3.1(+)-HA-Gγ-30-Flag-GST fusion

constructs were also created by PCR amplification of the HA-tagged Gγ gene from

pcDNA3.1(+)-HA-Gγ with the forward primers 5′-NheI-Gγ-10, 5′-NheI-Gγ-20,

5′-NheI-Gγ-30 and the reverse primer 3′-KpnI-Gγ, digestion with NheI and KpnI, and

ligation into pcDNA3.1(+)-Flag-GST. The pcDNA3.1(+)-HA-Gγ-frameshift-Flag-

GST and pcDNA3.1(+)-HA-Gγ(L15/E, L19/E)-Flag-GST fusion constructs were

created by PCR amplification of the HA-tagged Gγ-10 gene from pcDNA3.1(+)-HA-

Gγ-10-Flag-GST with the forward primers 5′-NheI-Gγ-10-fs, 5′-NheI-Gγ-10-L/E and

the reverse primer 3′-KpnI-G-gamma, digestion with NheI and KpnI, and ligation into

pcDNA3.1(+)-Flag-GST. The integrity of each construct was validated by automated

DNA sequencing and analysis. Table 1 provides the sequences of the primers used in

construction of these vectors.

5′-NheI-Gγ	TGGGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTG
5′-NheI-Gγ-10	TGGGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTGCCAGGAAGCTGGTAGAGCAGCTTAAGATG
5′-NheI-Gγ-20	TGGGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTGAAGCCAATATCGACAGGATAAAGGTGTCCAAGG
5′-NheI-Gγ-30	TGGGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTGCAGCTGCAGATTTGATGGCCTACTGTG

5′-NheI-Gγ-10-fs	GGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTGGCCAGGAAGCTGGTAGAGCAGCTAAAGATGAAGCCAATATCGACAGGATAAAGGTGTC
5′-NheI-Gγ-10-L/E	GGTT<u>GCTAGC</u>ATGTACCCATACGATGTTCCAGATTACGCTCCAGGAAGGAAGTAGAGCAGGAAAAGATGGAAGCCAATATCGACAGGATAAAGG
3′-KpnI-Gγ	TGGGTT<u>GGTACC</u>AAGGATGGCACAGAAAAACTTCTTCTCCCTAAACG
5'-BamHI-Flag-GST	AATTGG<u>GGATCC</u>ATGGATTACAAGGATGACGACGATAAGCAGGCGGGGAAGCCCATCCTC
3'-XhoI-GST	AATGTGCTCGAGCTAGGCCCTCAGCTCAGTGGGTGTATCTGGCTGCCGGCAGGGGTGAGACACCTGGAA
5'-BamHI-Flag –β-actin	AATTGG<u>GGATCC</u>ATGGATTACAAGGATGACGACGATAAGGATGATGATATCGCCGCGAATGTGCTCGAGTTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGAAGCATTTGCGGTGGACGATGGAGGGGCC

RNA Interference – Small interfering RNAs (siRNAs) were chemically synthesized (Dharmacon) to target nucleotides 345-365 of human PhLP1 (Lukov et al., 2005) and nucleotides 865-883 of human Gβ1 which are 100% conserved in Gγ2 (Krumins and Gilman, 2006). All the oligonucleotides had 3′dTdT overhangs. HEK293T cells were cultured in 12 well plates to 40-65% confluency. Cells were then transfected with the appropriate siRNA at 100 nM final concentration using Oligofectamine (Invitrogen) (Lukov et al., 2005). After 24 hours, the cells were transfected with 0.5 μg of the indicated cDNAs in the pcDNA3.1 (+) vector using Lipofectamine Plus reagent according to the manufacturer's protocol (Invitrogen). Cells were harvested for subsequent immunoprecipitation experiments 72 hours later. To assess the percent knockdown of specific proteins, 15 μg of cell lysate were immunoblotted with an anti-PhLP1antibody (Thulin et al., 1999) and anti-Gβ1-4 antibodies (Lee et al., 2004).

Translation Rate Determination - Transfected HEK-293 cells in 12-well plates were washed and incubated for 1 h in 1000 μl of methionine free media (Mediatech, Inc) supplemented with 4 mM L-glutamine (Sigma), 0.063g/L L-cystine dihydrochloride (USB) and 10% dialyzed fetal bovine serum (Invitrogen). The media was discarded and 400 μl of new media supplemented with 200 μCi/ml radiolabeled L-[^{35}S] methionine (Amersham Biosciences) was added. Cells were incubated at 23°C for 5, 10, 15, 25 and 35 min. to incorporate the [^{35}S] methionine into the newly synthesized proteins. After the pulse, the cells were washed and harvested for subsequent immunoprecipitation experiments.

For translation rate experiments involving the PhLP1 Δ1-75 dominant interfering mutant (Lukov et al., 2005), HEK 293T cells were plated in 12-well plates and grown to 70-80% confluency. The cells were then transfected using Lipofectamine Plus reagent according to the manufacturers protocol (Invitrogen). Wells were transfected with 1.0 μg each of either the empty vector control or PhLP1 Δ1-75-myc along with 1.0 μg each of either Flag-Gβ1 or HA-Gγ2 cDNAs. After 48 hrs, the cells were labeled with [^{35}S] methionine and prepared for immunoprecipitation as described below.

Immunoprecipitation -Transfected HEK 293T cells in 12-well plates were washed with phosphate-buffered saline (PBS, Fisher) and solubilized in immunoprecipitation buffer (PBS, pH 7.4, 0.5% NP-40 (Sigma), 0.6 mM PMSF, 6 μl/ml protease inhibitor cocktail per mL buffer (Sigma)). The lysates were passed through a 25-gauge needle 10 times and centrifuged at 14,000 rpm for 10 min. at 4ºC in an Eppendorf microfuge. The protein concentration for each sample was determined using the Coomassie Plus Protein Assay reagent (Thermo Scientific) and equal amounts of protein were used in the subsequent immunoprecipitations. 160 μg of total protein from the clarified lysates were incubated for 30 min. at 4°C with 2 μg anti-Flag (clone M2, Sigma) or anti-HA (clone 3F10, Roche) antibodies followed by an additional 25 μl of a 50% slurry of Protein A/G Plus agarose (Santa Cruz Biotechnology). The resulting mixture was incubated for 30 min. at 4°C as described (Lukov et al., 2005). The immunoprecipitated proteins were solubilized in SDS sample buffer and resolved on a 10 % Tris-glycine-SDS gel or a 16.5 % Tris-tricine-SDS gel for Gγ. The gels were dried on Whatman filter paper for subsequent

radioactivity measurements. Gels were visualized with a Storm 860 phosphor imager and the band intensities were quantified using the Image Quant software (GE Healthcare).

Quantitative RT-PCR - Total RNA was isolated using RNA-STAT60 (Tel-Test, Friendswood, TX) according to the manufacturer's protocol, except that following precipitation of the RNA with isopropanol, the centrifugation time was increased to 60 min. The resulting RNA preparation was treated with DNase I (DNA-*free*; Ambion, Inc., Austin, TX) to remove contaminating DNA. RNA from equal numbers of cells was reverse-transcribed using a dT oligo (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen). The cDNA template was then used for quantitative PCR (TaqMan) with the following primers and probe: human GAPDH, GNG2 and GNB1 (Applied Biosystem). The quantitative PCR conditions were as follows: 1 cycle at 50°C for 2 min and 1 cycle at 95°C for 10 min, followed by 60 cycles at 95°C for 15 s and 60°C for 1 min. The cell equivalents were based on GAPDH amplification.

Degradation Rate Determination - HEK-293T cells were treated with PhLP1 siRNA for 24 hrs and then transiently transfected with 0.5μg each of Flag-tagged Gβ1 and/or HA-tagged Gγ2 as indicated. After 72 additional hrs, the cells were pulsed with 200 μCi/ml radiolabeled L-[^{35}S] methionine (Amersham Biosciences) for 10 min followed by washing and chasing for the indicated time periods with 1 ml of media supplemented with 4 mM non-radiolabeled L-methionine (Sigma) and 4 μM of cycloheximide to stop the methionine incorporation. Cells were then lysed with the appropriate immunoprecipitation buffer (PBS, pH 7.4, 0.5% NP-40 (Sigma), 0.6 mM

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PMSF, 6 μl/ml protease inhibitor cocktail per mL buffer (Sigma)) and Gβ1 or Gγ2 was immunoprecipitated as described above. Immunoprecipitates were separated by SDS-PAGE and radioactive bands were visualized and quantified on the phosphor imager.

Assessment of Gβ and Gγ Aggregation - HEK-293 cells were treated with PhLP1 siRNA for 24 hrs and then transiently transfected with 0.5 μg each of Flag-Gβ1 and HA-tagged Gγ2. After 72 additional hrs, the cells were pulsed with 200 μCi/ml radiolabeled L-[^{35}S] methionine (Amersham Biosciences) for 25 min. Cells were then lysed with the appropriate immunoprecipitation buffer (PBS, pH 7.4, 0.5% NP-40 (Sigma), 0.6 mM PMSF, 6 μl/ml protease inhibitor cocktail per mL buffer (Sigma)) and Gβ1 or Gγ2 was immunoprecipitated as described above. Pellets obtained after centrifuging the lysed cells were resuspended in 0.5% SDS. Nascent proteins from the suspended pellets, immunoprecipitates and supernatant of the immunoprecipitate were separated by SDS-PAGE and radioactive bands were visualized and quantified on the phosphor imager.

Polysome Association Measurement - 100 mm dishes containing HEK293T cells were grown in Dulbecco's Modified Eagle's medium (Hyclone) supplemented with 10% fetal bovine serum (Sigma) to 100% confluency. The cells were incubated with or without 400 μM puromycin for 1 hr at 37°C after which the cells were incubated with 100 μg/ml cycloheximide for 15 min. to stabilize translating ribosomes (Raue et al., 2007). The cells were washed with 20 mM HEPES pH 7.5, 10 mM KCl, 1 mM EGTA, 5 mM MgCl, 10% glycerol, 2 mM β-mercaptoethanol (Hundley et al., 2005) and harvested in this same buffer + 0.2% NP-40 (Sigma). The

resulting lysate was passed through a 25-gauge needle 10 times after which it was centrifuged at 14,000 rpm for 10 min. at 4ºC in an Eppendorf microfuge to remove cell debris. The supernatant was loaded onto a 10-70% sucrose gradient containing 20 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$ and 2 mM dithiothreitol and centrifuged at 38,000 rpm for 4 hrs at 4ºC in a Beckman SW41 rotor. After the centrifugation, 500 µl fractions were collected and subjected to chloroform/methanol precipitation to concentrate the proteins and remove the sucrose. The precipitated proteins were solubilized in SDS-PAGE sample buffer and resolved on 10% Tris-Glycine-SDS gels for Gβ or 16.5% Tris-tricine gel for Gγ. The proteins were transferred to nitrocellulose and immunoblotted using an anti-CCTζ (Santa Cruz Biotechnology), anti-PhLP1 antibody (Thulin et al., 1999), or an anti-RPL23 antibody (Abgent). Immunoblots were incubated with the appropriate anti-rabbit, (Li-Cor Biosciences), or anti-rat (Rockland) secondary antibody conjugated with an infrared dye. Blots were scanned using an Odyssey Infrared Imaging System (Li-Cor Biosciences), and protein band intensities were quantified using the Odyssey software.

Results

In pulse/chase experiments designed to measure the effect of siRNA-mediated PhLP1 knockdown on Gβγ assembly, an ~50% decrease in the amount of Gβ and Gγ synthesized during the 10 min. pulse with [35S] methionine was consistently observed (Lukov et al., 2005). To confirm and further characterize this decrease, the effect of PhLP1 knockdown on the rate of Gβ and Gγ synthesis was

measured. HEK-293T cells were depleted of 80% of their endogenous PhLP1 (Fig.

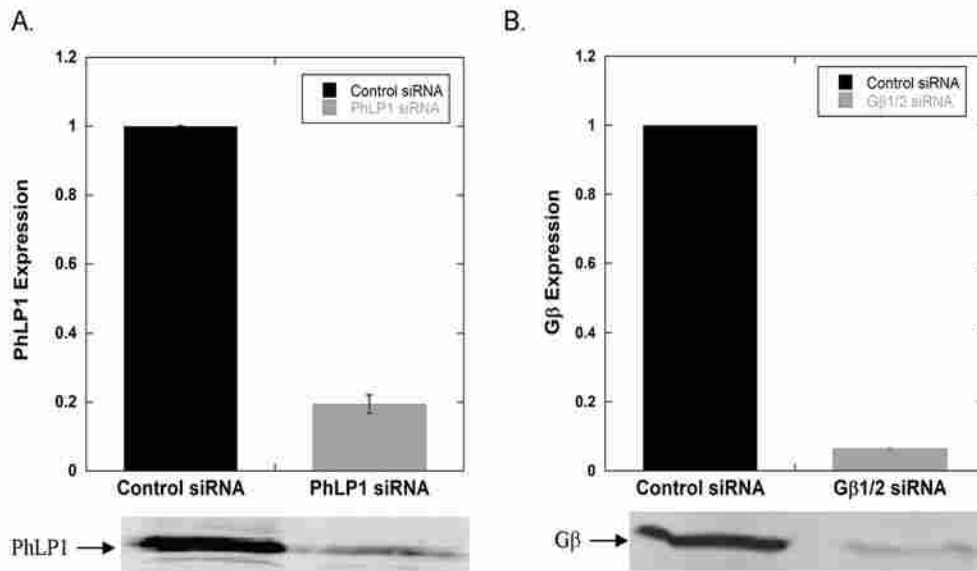2-1A) and were subsequently transfected with Gβ and Gγ together or individually.

Figure 2-1. siRNA inhibition of PhLP1 and Gβ1 expression.
HEK-293T cells were treated with an siRNA targeting nucleotides 345-365 of PhLP1 (A), nucleotides
865- 883 of Gβ1/2 (B) or a scrambled control siRNA as indicated. After 96 hrs, the cells were lysed and
the extracts were immunoblotted for PhLP1 or Gβ1. Blots were visualized and quantified using an
Odyssey Infrared Imaging System (LiCor Biosciences). Bars represent the average ± the standard error
from three experiments. Representative blots are shown below the graphs.

The rate of Gβ and Gγ synthesis was then measured by [^{35}S] methionine labeling for

increasing times, followed by immunoprecipitation of the nascent Gβ and

quantification of the amounts synthesized at each time point. When Gβ and Gγ were

co-expressed, the rate of Gγ synthesis was decreased 55% by PhLP1 depletion (Fig.

2-2A). A very similar 54% decrease was observed when Gγ was over-expressed in

the absence of Gβ (Fig. 2-2B). The effects of PhLP1 depletion were less pronounced

in the case of Gβ. When Gβ and Gγ were co-expressed, the rate of Gβ synthesis was

decreased by 37% (Fig. 2-2C), but was unchanged when Gβ was expressed in the

absence of Gγ (Fig. 2-2D). These results confirm the initial observations and reveal

important differences between the effects of PhLP1 depletion on Gβ and Gγ
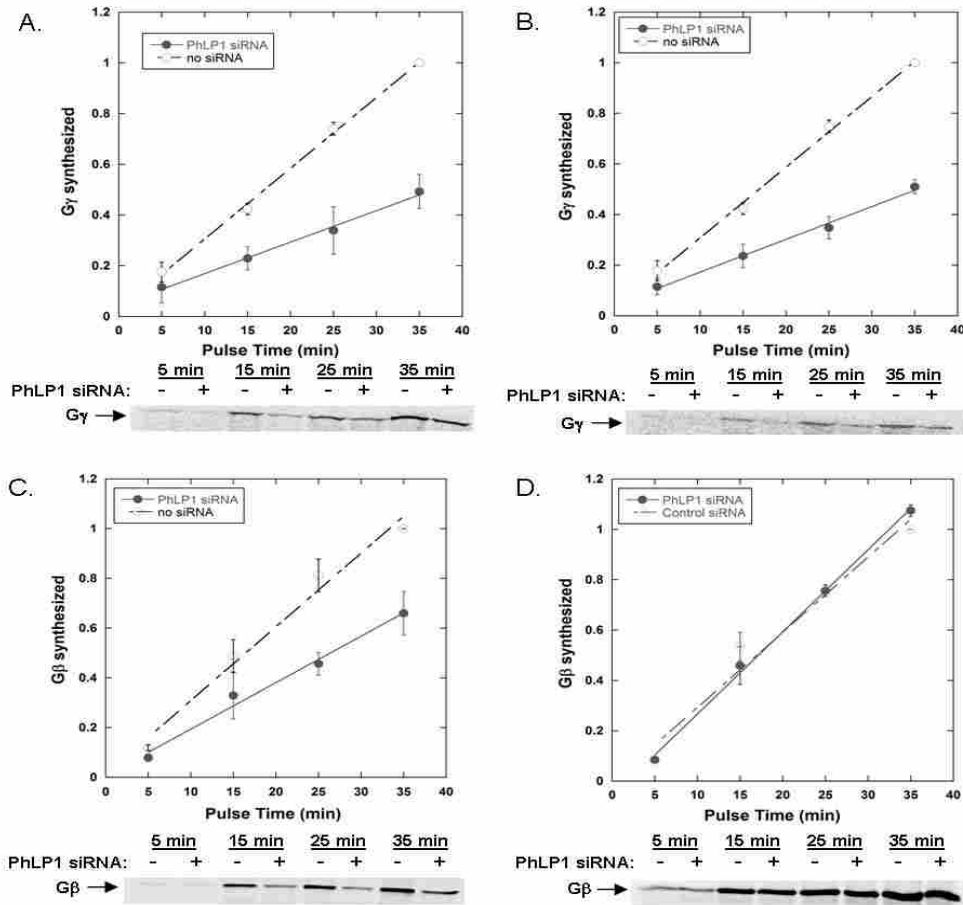
synthesis.

Figure 2-2. PhLP1 knockdown inhibits the rate of Gγ synthesis.
HEK-293T cells were treated with or without PhLP1 siRNA for 24 hrs and then transiently
transfected with FLAG-tagged Gβ1 and HA-tagged Gγ2 (A and C), HA-Gγ2 alone (B), or FLAG-
Gβ1 alone (D). After 48 additional hrs, the cells were pulsed with ^{35}S-methionine for the times
indicated, then lysed and immunoprecipitated with anti-HA for Gγ (A and B) or anti-FLAG for Gβ
(C and D). Immunoprecipitates were separated by SDS-PAGE and radioactive Gγ or Gβ bands were
visualized and quantified on a phosphor imager. Data points represent the average ± the standard
error from three experiments. Representative gels are shown below the graphs.

The fact that PhLP1 depletion did not change the rate of Gβ synthesis in the

absence of Gγ suggested that the effect was specific and not simply a result of double

stranded RNA-induced inhibition of overall translation (Hovanessian, 2007). To

further assess specificity, the rate of glutathione-S-transferase (GST) synthesis, when

expressed from the same expression vector as Gβ and Gγ, was also tested with and

without PhLP1 siRNA and showed no difference (Fig.2-3A). In addition, the rate of

endogenous actin synthesis was also measured with and without PhLP1 siRNA and

again there was no change (Fig.2-3B). These results indicate that siRNA-mediated

PhLP1 depletion specifically inhibited Gγ synthesis without affecting protein

synthesis in general. Another way to disrupt Gβγ dimer formation is through the

PhLP1Δ1-75 truncation, which acts in a dominant negative manner to block Gβγ

assembly. PhLP1Δ1-75 competes with endogenous PhLP1 by forming a PhLP1Δ1-

75-Gβ-CCT complex that does not release Gβ to interact with Gγ (Lukov et al.,

2006; Lukov et al., 2005). The effect of this PhLP1 variant on the rate of Gγ and Gβ

synthesis was also measured. Over-expression of PhLP1Δ1-75 caused a 45%

reduction in Gγ synthesis whether co-expressed with Gβ or not (Fig. 2-4A and B). In

contrast, Gβ expression was only decreased 20% by PhLP1Δ1-75 when co-expressed

with Gγ (Fig. 2-4C) and there was no effect of PhLP1Δ1-75 when Gβ was expressed

in the absence of Gγ (Fig. 2-4D). These results confirm the siRNA results and further

indicate that when PhLP1 function is lost, there is a significant decrease in the rate of

Gγ synthesis and a modest decrease in Gβ synthesis, but only in the presence of Gγ.

Figure 2-3. PhLP1 depletion has no effect on GST or actin synthesis.
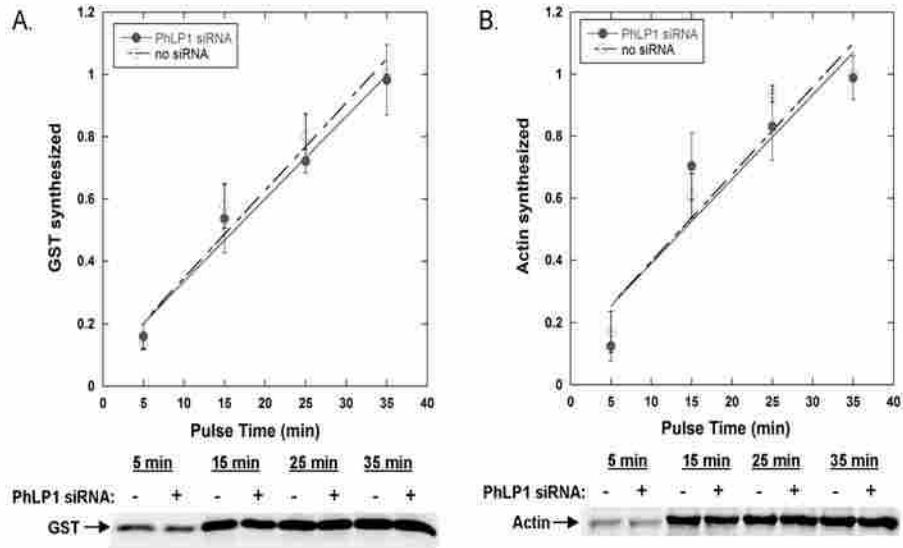HEK-293T cells were treated with or without PhLP1 siRNA for 24 hrs and then transiently transfected with GST (A) or left untransfected (B). After 72 additional hrs, the cells were pulsed with ^{35}S-methionine for the times indicated, then lysed and immunoprecipitated with anti-GST (A), or endogenous nascent β-actin was precipitated with anti-Flag (B). Precipitates were separated by SDS-PAGE and radioactive GST or β-actin bands were visualized and quantified on a phosphor imager. Data points represent the average ± the standard error from three experiments. Representative gels are shown below the graphs.

There are several ways in which the apparent rate of Gβ and Gγ synthesis could be

decreased by PhLP1 depletion. An obvious way would be the rapid degradation of

the unassembled Gβ and Gγ in the absence of PhLP1. This possibility was tested by

measuring the rate of Gβ and Gγ degradation with and without PhLP1 siRNA in a

pulse-chase experimental format. Unexpectedly, there was no significant difference

in the rate of degradation of either Gβ or Gγ under these conditions despite the fact

that PhLP1 depletion resulted in less Gβ and Gγ synthesized during the pulse phase of

the experiment (Fig. 2-5A and B). All of the curves showed a degradation sensitive

fraction that decayed with $t_{1/2}$ values in the 5-10 min range and leveled off after about

60 min., leaving a stable, degradation-resistant fraction. A second possible reason for

the decreased rate of Gβ and Gγ synthesis could be the increased degradation of their

mRNAs in the absence of PhLP1. This possibility seemed unlikely given that the

mRNAs were produced from expression vectors with non-native 5′ and 3′

untranslated regions (UTR) that normally carry the regulatory elements in native

mRNAs (Hentze et al., 2007). Accordingly, RT-PCR measurements showed no
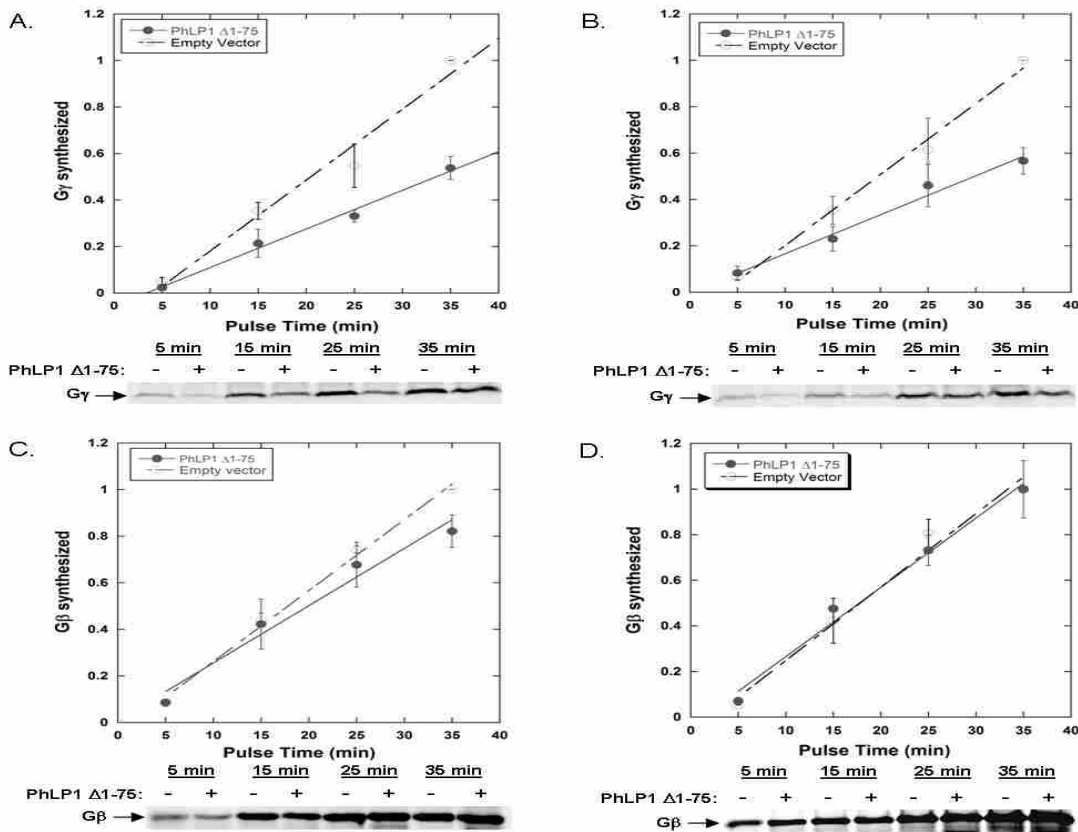
Figure 2-4. PhLP1 Δ1-75 inhibits the rate of Gγ synthesis.
HEK-293T cells were transiently transfected with PhLP1 Δ1-75 along with FLAG-Gβ1 and HA-Gγ2 (A and C), or HA-Gγ2 alone (B), or FLAG-Gβ1 alone (D). After 48 hrs, the cells were pulsed with ^{35}S-methionine for the times indicated, then lysed and immunoprecipitated with anti-HA for Gγ (A and B) or anti-FLAG for Gβ (C and D). Immunoprecipitates were separated by SDS-PAGE and radioactive Gγ or Gβ bands were visualized and quantified on a phosphor imager. Data points represent the average ± the standard error from three experiments. Representative gels are shown below the graphs.

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difference in Gβ or Gγ mRNA in the presence or absence of PhLP1 siRNA, confirming that mRNA levels of Gβ and Gγ do not change in the absence of PhLP1 (Fig. 2-6).

Finally, it is possible that the decrease in Gβ and Gγ synthesis upon PhLP1 depletion could be caused by aggregation of the newly synthesized proteins into insoluble aggregates that are lost during the immunoprecipitation process. This possibility was tested by assessing the amount of ^{35}S-labeled Gβ and Gγ in each immunoprecipitation fraction by SDS-PAGE. If the nascent proteins were forming insoluble aggregates, they would be found in the pellet after centrifugation of the initial cell extract. However, no Gβ or Gγ was found in this pellet or in the supernatant after immunoprecipitation, but only in the immunoprecipitate itself in both depleted or control cells, indicating that no aggregation was occurring (Fig. 2-5 C and D). This process of elimination leads to the unexpected conclusion that the loss of PhLP1 function somehow causes a decrease in the rate of translation of the Gβ and Gγ polypeptides. It is interesting to note that Gγ translation was more sensitive to PhLP1 knockdown than Gβ translation despite the fact that PhLP1 is known to interact with nascent Gβ but not nascent Gγ (Lukov et al., 2005). In fact, Gγ translation was inhibited by PhLP1 knockdown to the same degree in the presence or absence of Gβ co-expression, whereas Gβ translation was only sensitive to PhLP1 knockdown when Gγ was co-expressed (Fig. 2-2). These observations suggest that PhLP1 contributes specifically to the translation of Gγ. To achieve this specificity, some part of the mRNA or amino acid sequence of Gγ must be recognized and
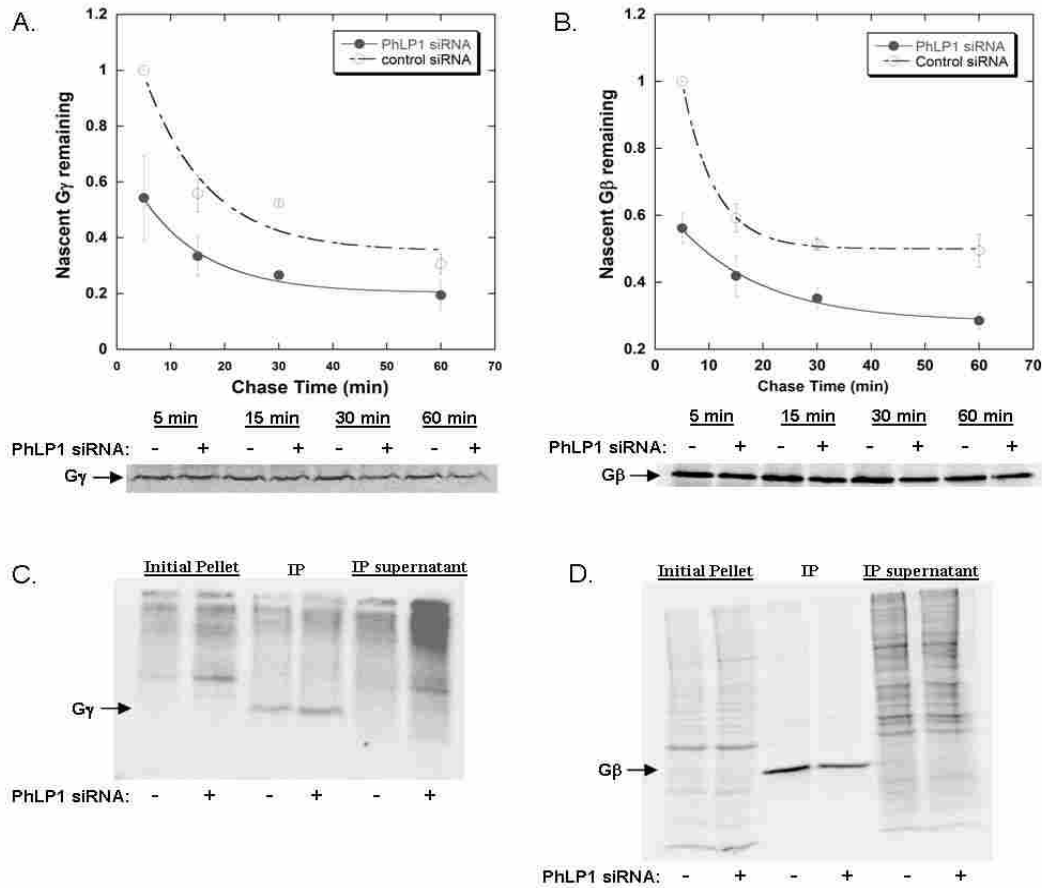
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Figure 2-5. PhLP1 knockdown does not affect Gγ and Gβ degradation and aggregation.
HEK-293 cells were treated with PhLP siRNA for 24 hrs and then transiently transfected with FLAG-Gβ1 and HA-Gγ2. After 48 additional hrs, degradation experiments (A and B) were initiated by pulsing the cells with ^{35}S-methionine for 10 min and then chasing with unlabeled methionine and cycloheximide for the indicated times. Cells were then lysed and immunoprecipitated with anti-HA for Gγ (A) or anti-FLAG for Gβ (B). Immunoprecipitates were separated by SDS-PAGE and radioactive Gγ or Gβ bands were visualized and quantified on a phosphor imager. Data points represent the average ± the standard error from three experiments. Lines represent a non-linear least squares fit of the data to a first order rate of degradation equation. Representative gels are shown below the graphs.

Aggregation experiments (C and D) were initiated by pulsing the cells with ^{35}S-methionine for 25 min, lysing the cells and pelleting insoluble material. The initial cell pellet was resuspended in 0.5% SDS for SDS-PAGE analysis. The corresponding supernatant was immunoprecipitated with anti-HA for Gγ (C) or anti-FLAG for Gβ (D). The initial cell pellet, the immunoprecipitate (IP) and the supernatant after immunoprecipitation were all subjected to SDS-PAGE and radioactive bands were visualized on a phosphor imager. Gels shown are representative of at least three experiments.

translation inhibited in the absence of PhLP1. Translational initiation of RNA

transcipts is often controlled by their 5′ and 3′ UTRs (Dever, 2002; Hentze et al.,
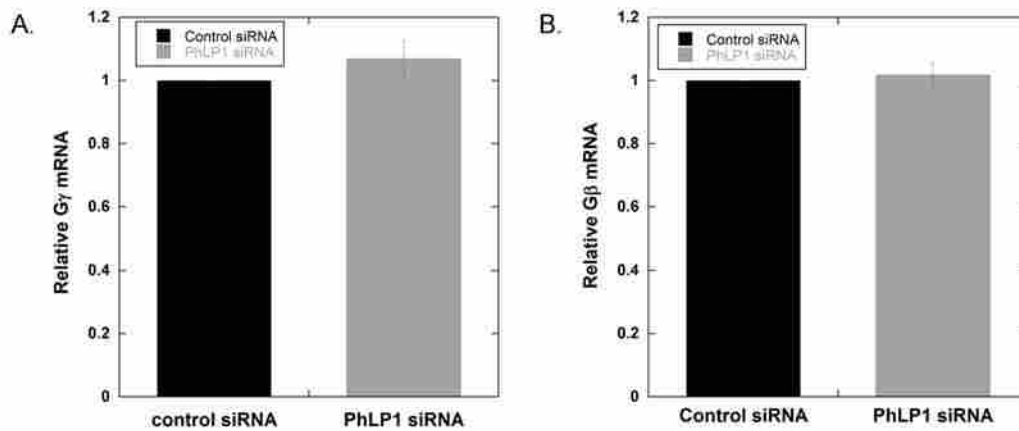
2007).

Figure 2-6. PhLP1 depletion has no effect on Gγ or Gβ mRNA levels.
HEK-293 cells were treated with PhLP1 or control siRNA for 24 hrs and then transiently
transfected with HA-Gγ2 and FLAG-Gβ1. After an additional 48 hrs, the cells were lysed, total
RNA was isolated, and the mRNA was reverse transcribed. The resulting cDNA was used as a
template for quantitative RT-PCR with Gγ2 and Gβ1 specific probes. The RT-PCR data were
normalized to the control siRNA values. Bars represent the average ± the standard error from three
experiments.

However, in these experiments the 5′ and 3′ UTRs of Gγ come from the expression

vector and are exactly the same as the GST construct whose translation was

unaffected by PhLP1 depletion. Thus, it is unlikely that the UTRs provide the

observed specificity. Sequences within the coding region of the mRNA transcript

could provide the observed specificity, but this is uncommon (Hentze et al., 2007).

Another way to specifically control Gγ translation would be to recognize the N-

terminal amino acid sequence of Gγ as it emerges from the ribosome exit tunnel in

such a manner that translation stalls in the absence of PhLP1. Such is the case with

the signal recognition particle that detects N-terminal leader sequences of membrane

and secreted proteins co-translationally and inhibits the ribosome until the signal

recognition particle binds its receptor and translocon in the endoplasmic reticulum membrane (Halic and Beckmann, 2005; Shan and Walter, 2005; Walter and Blobel, 1981). This latter idea was tested by preparing a fusion construct of Gγ with GST attached to its C-terminus. Various N-terminal truncations of the Gγ sequence were prepared and tested for inhibition of translation in the absence of PhLP1 (Fig. 2-7A). Fusing Gγ to GST made GST translation as sensitive to PhLP1 depletion as Gγ itself, confirming that the Gγ sequence was sufficient to confer PhLP1 sensitivity to translation. Deletion of amino acids 1-10 of Gγ did not change the PhLP1 sensitivity. However, deletion of amino acids 1-20 or 1-30 of Gγ created constructs whose translation was totally insensitive to PhLP depletion. This result suggests that the observed inhibition of Gγ translation upon PhLP depletion is mediated by a factor that recognizes the sequence between residues 11-20 of Gγ as it leaves the ribosome exit tunnel. To further test this possibility, the effect of PhLP1 knockdown on the translation of a construct containing residues 11-20 of Gγ fused to the N-terminus of GST was also measured. PhLP1 knockdown decreased the rate of translation of this construct by nearly the same amount as the full-length Gγ-GST construct (33% compared to 40%, respectively), indicating that the sequence between residues 11-20 of Gγ was sufficient for PhLP1-dependent translation (Fig. 2-7B).

These data point to the amino acid sequence of Gγ 11-20 as the determining factor in PhLP1-dependent translation inhibition. However, they do not rule out the possibility that the Gγ mRNA nucleotide sequence of codons 11-20 is responsible. To test this possibility, a Gγ construct was designed in which the nucleotide sequence

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of residues 11-20 was frame-shifted by inserting one nucleotide prior to codon 11 and then removing one nucleotide after codon 20 to restore the reading frame at codon 21 (Fig. 2-7C). This modification retains the mRNA sequence of codons 11-20, but scrambles the amino acid sequence. If the mRNA sequence was responsible for PhLP1 sensitivity, the one nucleotide insertion would have little or no effect on translation, but if the amino acid sequence were responsible, the scrambling of residues Gγ 11-20 would make Gγ translation insensitive to PhLP1 depletion. The data of Fig. 2-7C show that the rate of translation of this frame-shifted Gγ construct was completely insensitive to PhLP1 knockdown, confirming that it is the amino acid sequence of Gγ residues 11-20 that confers PhLP1-sensitivity to Gγ translation. The intriguing interpretation of this finding is that the N-terminus of actively translating Gγ somehow depends on PhLP1 to complete Gγ translation. If PhLP1 is absent, the translation process stalls.

A potential insight into the mechanism of this apparent co-translational regulation of Gγ synthesis is that residues 11-20 of Gγ form a coiled-coil interaction with the N-terminus of the Gβ subunit, making extensive contacts (Sondek et al., 1996; Wall et al., 1995). This observation points to a role for Gβ in the regulation of Gγ translation. This possibility could be tested by making amino acid substitutions of Gγ in the 11-20 sequence that disrupt its coiled-coil interaction with
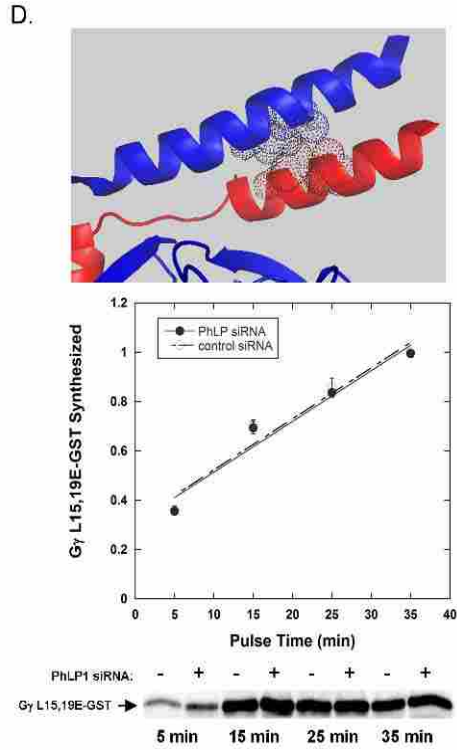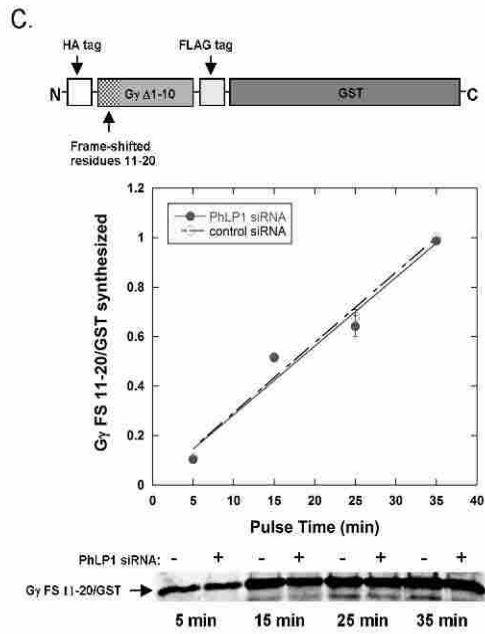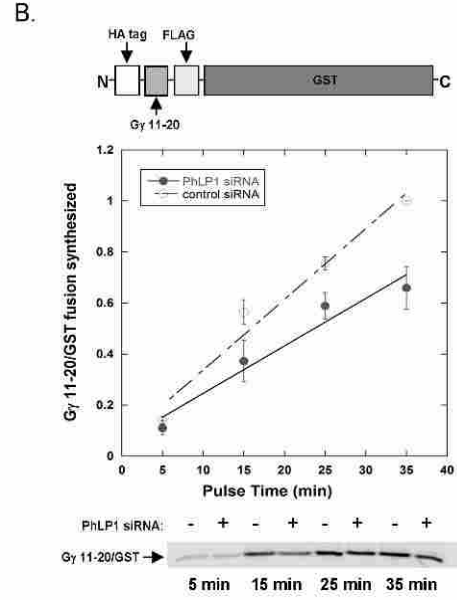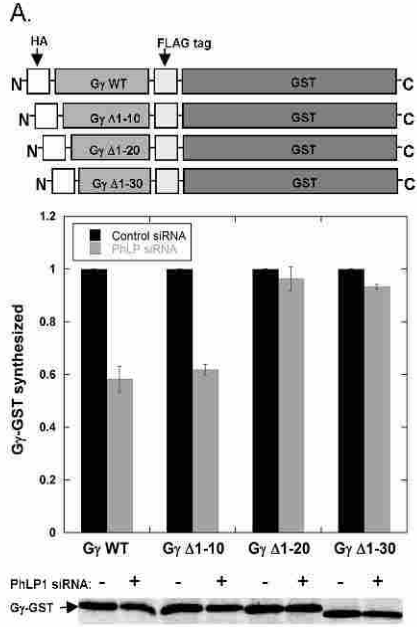
A.

B.

C.

D.

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Figure 2-1. Residues 11-20 of Gγ mediate the inhibitory effects of PhLP1 knockdown.
A) The effect of PhLP1 depletion on the translation of Gγ2-GST constructs with N-terminal Gγ2 truncations is shown. A schematic of the constructs used in this experiment is found about the graph. The N-terminus of each construct was capped by an HA tag and a FLAG sequence was used as a linker between Gγ2 and GST as shown in the diagram. HEK-293T cells were treated with PhLP siRNA for 24 hrs and then transiently transfected with the Gγ2-GST constructs as indicated. After 72 additional hrs, the cells were pulsed for 35 min with $[^{35}S]$ methionine and the fusion proteins were immunoprecipitated with an anti-HA antibody. Nascent Gγ2-GST in the immunoprecipitates was quantified as in Fig. 2-1. Bars represent the average ± the standard error from three experiments and a representative gel is shown below the graph. B) The effect of PhLP1 depletion on the translation of a fusion construct of Gγ residues 11-20 and GST is shown. A schematic of the construct is found about the graph. HEK-293T cells were treated with siRNA, transfected with this construct and translation was measured as in Fig.2-2. Data points represent the average ± the standard error from three experiments and a representative gel is shown below the graph. C) The effect of PhLP1 depletion on the translation of a Gγ construct in which a frameshift (FS) was introduced into residues 11-20 is shown. The Gγ 11-20 frameshift was created by adding a single nucleotide to the 5′ end of codon 11 of the Gγ Δ1-10/GST construct to shift the reading frame and a single nucleotide was removed from the 3′ end of codon 20 to restore the correct reading frame at residue 21. HEK-293T cells were treated with PhLP1 siRNA, transfected with this construct and translation was measured as in Fig.2-2. Data points represent the average ± the standard error from three experiments and a representative gel is shown below the graph. D) The effect of PhLP1 depletion on the translation of a Gγ construct in which leucine residues L15 and L19 from the Gγ Δ1-10/GST construct were substituted with glutamate is shown. These substitutions disrupt the hydrophobic interaction between L15 and L19 of Gγ (red dotted spheres) and residues A11 and L14 of Gβ (blue dotted spheres) that contribute to the coiled-coil interaction between the N-termini of Gγ (red) and Gβ (blue). HEK-293T cells were treated with PhLP1 siRNA, transfected with this construct and translation was measured as in Fig.2-2. Data points represent the average ± the standard error from three experiments and a representative gel is shown below the graph. The ribbon diagram was made from the $G\alpha_i\beta_1\gamma_2$ X-ray crystal structure (PDB ID: 1GP2) (Wall et al., 1995) using PyMol.

Gβ and measure the effect of PhLP1-depletion on this Gγ variant. Leucines 15 and

19 of Gγ2 are conserved residues that make extensive contacts with Gβ1 in the

coiled-coil (Wall et al., 1995) (Fig. 2-7D). Consequently, these residues were

substituted with glutamate, and the effect of PhLP1 depletion on the translation of the

GγL15E/L19E variant was measured. The rate of translation of the variant was

completely insensitive to PhLP1 knockdown (Fig. 2-7D), indicating that the coiled-

coil interaction between the N-terminal regions of Gβ and Gγ is necessary to elicit

stalling of Gγ translation in the absence of PhLP1.

To further explore the role of Gβ in Gγ translation, the effects of both cellular depletion and over-expression of Gβ on Gγ translation were measured without changing the endogenous levels of PhLP1. Interestingly, siRNA-mediated Gβ depletion resulted in a significant increase in the rate of Gγ translation (Fig. 2-8A), the opposite of what was observed with PhLP1 knockdown. Likewise, the over-expression of Gβ caused a decrease in Gγ translation (Fig. 2-8B). These reciprocal effects of Gβ lead to the unexpected conclusion that the association of Gβ with Gγ must inhibit the rate of Gγ translation.
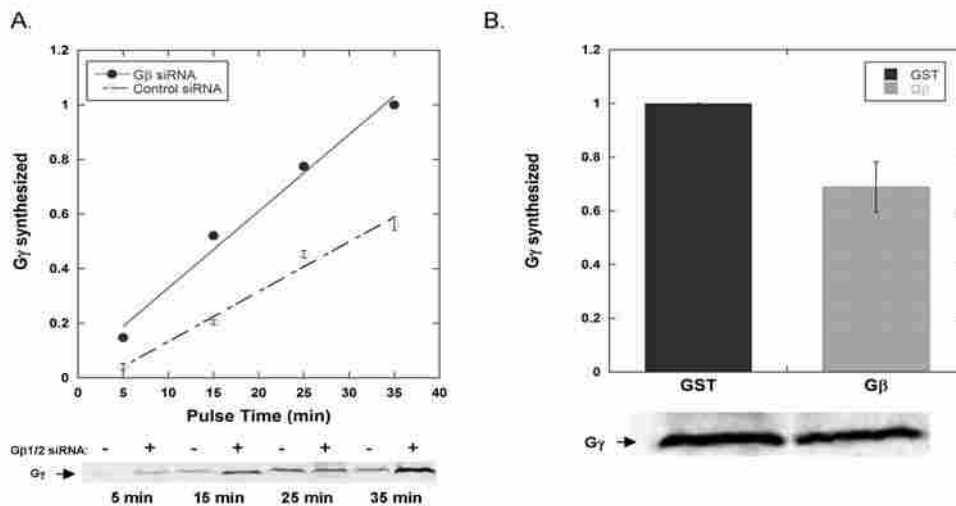
Figure 2-2. Effects of Gβ knockdown and over-expression on the rate of Gγ synthesis.
HEK-293T cells were treated with or without an siRNA targeting both Gβ1 and Gβ2 for 24 hrs and then transiently transfected with HA-tagged Gγ2. After 72 additional hrs, the rate of Gγ translation was measured as in Fig.2- 1. Data points represent the average ± the standard error from three experiments. Error bars that are not visible are smaller than the symbol. A representative gel is shown below the graph. B. HEK-293T cells were transiently transfected with HA-Gγ and either Gβ or a GST control. After 48 hrs, the cells were pulsed for 35 min with [^{35}S] methionine and Gγ translation was measured as in Fig.2-2. Bars represent the average ± the standard error from three experiments and a representative gel is shown below the graph.

The results presented thus far point to a unique co-translational mechanism for the regulation of Gγ synthesis in which formation of the N-terminal coiled-coil with Gβ stalls Gγ translation on the ribosome, while PhLP1 somehow unblocks the stalled ribosome and allows translation to continue. For this regulation to occur, Gβ must be able to interact with the N-terminus of Gγ as it exits the ribosome. However, Gβ is not free in the cytosol prior to its association with Gγ but is bound to CCT (Lukov et al., 2006; Wells et al., 2006). Interestingly, CCT has been previously shown to interact co-translationally with nascent polypeptides on actively translating ribosomes (Etchells et al., 2005; McCallum et al., 2000). Thus, it is possible that a ribosome associated Gβ-CCT complex could interact with the nascent Gγ and stall Gγ translation. This idea is attractive because it also provides a means for PhLP1 to unblock Gγ translation. PhLP1 has been shown to release Gβ from CCT and permit its subsequent association with Gγ (Lukov et al., 2006; Lukov et al., 2005). By releasing Gβ, PhLP1 may relieve its inhibition of Gγ synthesis. This hypothesis predicts that PhLP1 and CCT would be associated with actively translating ribosomes. To test this prediction, polysomes were isolated from cell extracts by sucrose gradient centrifugation and were immunoblotted for the presence of PhLP1 and CCT. Both were found in significant amounts in the high density polysomal fractions on the sucrose gradient (Fig. 2-9). The 60S ribosomal protein L23 was also found in these same fractions, confirming that they contained ribosomes. Pretreatment of cells with puromycin, an antibiotic that blocks translation and dissociates polysomes into 40S and 60S ribosomal subunits (Blobel and Sabatini, 1971), resulted in a nearly complete shift of PhLP1 and CCT to the low density

cytosolic fractions, while L23 shifted to intermediate fractions corresponding to the

migration of the 60S subunit as expected (Fig. 2-9). These results demonstrate that

PhLP1 and CCT are associated with polysomes and could thus interact co-

translationally with Gγ.
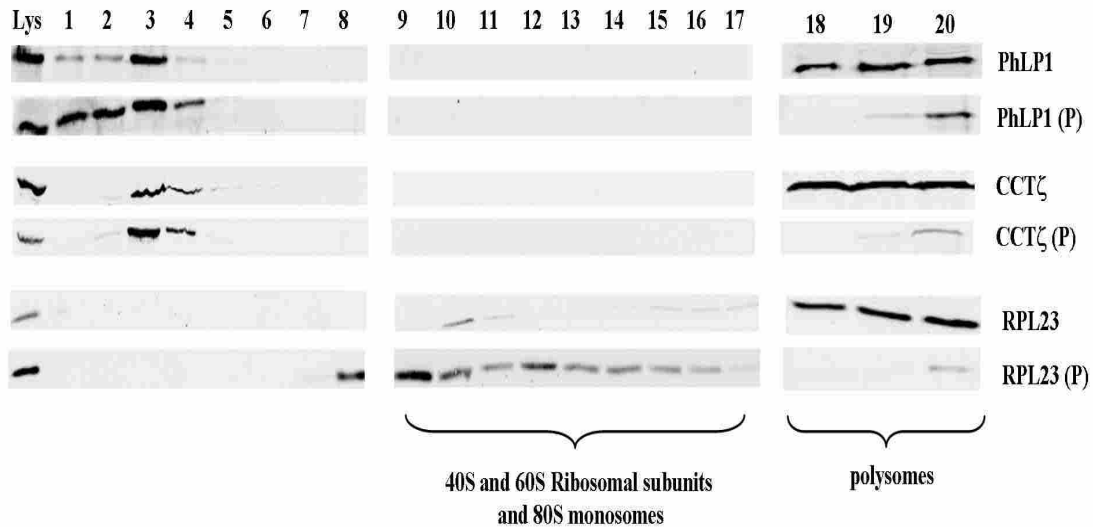
Figure 2-3. Association of PhLP1 and CCT with polysomes.
Binding of PhLP1 and CCT to polysomes was measured by sucrose gradient centrifugation. HEK-293
cells were incubated with or without puromycin (P), a translation inhibitor that disrupts polysomes, and
then extracted with detergent. Cell lysates were separated by high speed centrifugation through 10-70%
sucrose gradients. Fractions were collected from the top to bottom of the gradient and immunoblotted for
PhLP1, CCTζ or the 60S ribosomal protein subunit RPL23. Results are representative of three separate
experiments.

Discussion

For many years since the discovery of the Gβγ complex, a question has

persisted regarding how the two nascent polypeptides are brought together when

neither of them can form a stable structure on their own. Recent work has shown that

CCT and PhLP1 are essential chaperones in the folding of Gβ and in its subsequent

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association with Gγ (Humrich et al., 2005; Knol et al., 2005; Lukov et al., 2006; Lukov et al., 2005). CCT binds nascent Gβ and assists in the formation of the Gβ seven-bladed β-propeller structure (Lukov et al., 2006; Wells et al., 2006). PhLP1 associates with Gβ in the CCT folding cavity and catalyzes the release of Gβ from CCT and its subsequent interaction with Gγ (Lukov et al., 2006; Lukov et al., 2005). However, current data provide little insight into the mechanism by which Gγ associates with Gβ. It is clear that Gγ does not form its high affinity complex with Gβ in the CCT folding cavity because no interaction of Gγ with Gβ-CCT complexes has been observed (Lukov et al., 2006; Wells et al., 2006). It seems likely that Gγ only makes full contact with Gβ after PhLP1 has released Gβ from CCT. Once released, Gγ can readily associate with Gβ bound to PhLP1 because the Gγ binding site on Gβ does not overlap the PhLP1 binding site (Gaudet et al., 1996). However, little is known about the status of Gγ prior to its assembly with Gβ. One report has shown that Gγ interacts with the J-domain containing chaperone DRiP78 (Dupre et al., 2007b), and siRNA-mediated depletion of DRiP78 reduced Gβγ dimer formation, suggesting that DRiP78 is involved in Gβγ assembly.

The results presented here point to a novel co-translational mechanism of Gβγ assembly. The data show that the rate of Gγ translation is sensitive to PhLP1 activity in the cell. Cellular depletion of PhLP1 by siRNA or over-expression of a dominant-negative PhLP Δ1-75 variant, which both block Gβγ assembly (Lukov et al., 2005), reduced the rate of Gγ translation ~ 2-fold. This effect of PhLP depletion was dependent on the ability of the N-terminus of Gγ to form its coiled-coil interaction

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with the N-terminus of Gβ. Moreover, siRNA-mediated depletion of Gβ enhanced the rate of Gγ translation and over-expression of Gβ decreased Gγ translation. These results lead to the unexpected conclusion that formation of the coiled-coil interaction between Gβ and Gγ inhibits the rate of Gγ translation. To inhibit translation, this interaction must occur co-translationally which requires the Gβ-CCT complex to be associated with translating ribosomes. Indeed CCT and PhLP1 were both found associated with polysomes, making possible a co-translational interaction between Gβ and Gγ on the ribosome.

A model for PhLP1-mediated co-translational Gβγ assembly that is consistent with all the current data is depicted in Fig. 2-10. In this model, the Gγ N-terminus associates with the N-terminus of Gβ in the CCT folding cavity forming the coiled-coil interaction as Gγ emerges from the ribosome exit tunnel. This interaction stalls further translation of the Gγ mRNA transcript until PhLP1 is available to release Gβ from CCT and relieve the inhibition. Once Gβ is released, translation continues and the C-terminal amino acids of Gγ find their contacts along the surface of the Gβ propeller opposite the PhLP1 binding site as they emerge from the exit tunnel.

There are several advantages to a co-translational mechanism for Gβγ assembly. First, Gγ makes extensive hydrophobic interactions with Gβ all along its entire 70 amino acid length. If Gγ were synthesized and released into the cytosol where the total protein concentration is high, the likelihood of Gγ finding Gβ and forming the Gβγ dimer prior to its aggregation with other proteins would be very low. Second, the binding of Gγ to chaperones like DRiP78, may make Gβγ dimer

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formation difficult. The efficiency of Gβγ assembly from chaperones could be low

because of the numerous hydrophobic contacts with the chaperone that would need to

be broken and then reformed with Gβ. Third, by assembling the Gβγ dimer co-

translationally, the hydrophobic amino acids of Gγ would associate immediately with

their sites on Gβ as they emerged from the exit tunnel, avoiding problems with

aggregation or transfer from chaperones.
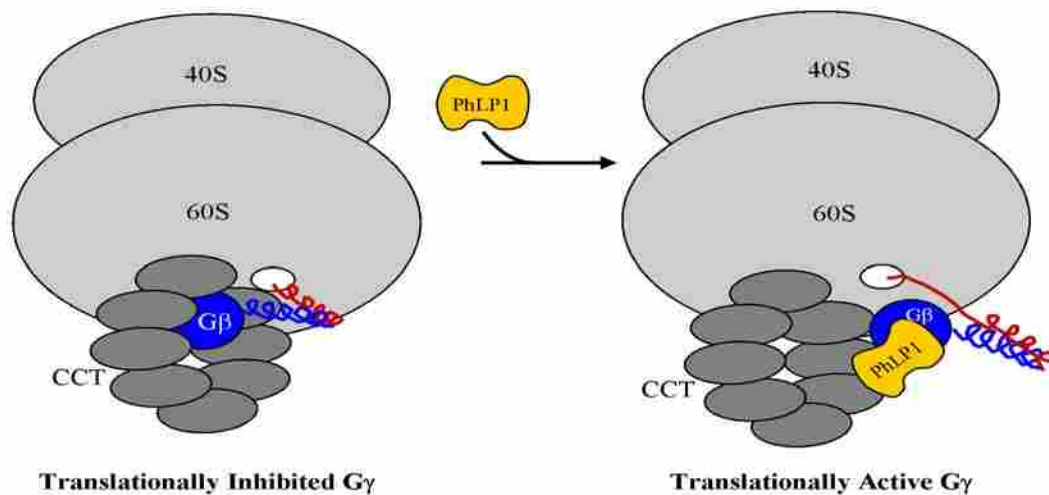
Figure 2-4. Model of PhLP1-mediated co-translational assembly of Gγ with Gβ.
The model depicts the proposed translational inhibition of Gγ as its N-terminus emerges from the ribosome and forms a coiled-coil interaction with the N-terminus of Gβ in the CCT complex. Gγ translation is stalled until PhLP1 interacts with Gβ and releases it from CCT. In the presence of PhLP1, Gγ translation resumes and the nascent Gγ interacts co-translationally with Gβ, forming the Gβγ dimer.

There are two intriguing questions raised by these results. First, why would Gγ translation stall in the presence of its Gβ binding partner? If anything, one would predict that Gγ translation would be accelerated by Gβ. The reason for stalling appears to be the need to wait for PhLP1 to associate with the Gβ-CCT complex and release Gβ. The inability of Gγ to form its high affinity complex with Gβ on CCT (Lukov et al., 2006; Wells et al., 2006) indicates that most of the Gγ contacts on Gβ are masked by CCT. If Gγ translation were to continue before these contacts could be made, then many hydrophobic residues of Gγ would be left exposed and subject to aggregation or binding by chaperones of the degradative pathway (Kaganovich et al., 2008). PhLP1 is the limiting factor. It is expressed at an ~4-fold lower concentration than CCT (McLaughlin et al., 2002b), thus the cell would have many Gβ-CCT complexes not associated with PhLP1. These complexes would co-translationally bind the N-terminus of nascent Gγ in the coiled-coil interaction and stall translation until PhLP1 could associate with Gβ and release it from CCT, freeing up the hydrophobic contact sites for Gγ on Gβ and allowing translation to resume. In this manner, exposure of the hydrophobic residues of both Gβ and Gγ would be minimized.

The second question concerns the mechanism of stalling. How does formation of the Gγ N-terminal coiled-coil with Gβ cause Gγ translation to stall until PhLP1 can release Gβ from CCT? Insight can be gained from other examples of co-translational ribosome stalling. In the case of the eukaryotic signal recognition

particle (SRP), the signal peptide is recognized by the SRP54M domain at the exit tunnel (Halic et al., 2006), which positions the SRP Alu domain in the elongation factor binding site where it would inhibit elongation through steric hindrance (Halic et al., 2004). It is possible that co-translational binding of the Gγ N-terminus to Gβ in the CCT complex positions CCT so that it blocks elongation factor binding. The distance between the exit tunnel and the elongation factor binding site is 12 nm (Halic et al., 2004) while the diameter of the CCT complex is 15 nm (Llorca et al., 1999). Thus, the CCT complex is sufficiently large to block the elongation factor binding site if positioned correctly. Alternatively, co-translational formation of the coiled-coil between nascent Gγ and Gβ may cause a conformational change in the nascent Gγ that is transferred up the exit tunnel and into the peptidyl transferase center, disrupting translation. An example of this type of translational inhibition is seen with the TnaC leader peptide of the E. coli typtophanase operon, in which high concentrations of tryptophan cause changes in the interactions between the leader peptide in the ribosome exit tunnel that disrupt the peptidyl transferase center (Seidelt et al., 2009). Additional structural work will be required to determine the molecular details of Gβ-mediated stalling of Gγ translation.

In summary, this work outlines an elegant mechanism that links translation of the Gγ subunit to formation of the Gβγ dimer, bringing together the unstable Gβ and Gγ polypeptide chains in a way that avoids aggregation or degradation of the Gγ subunit. It will be of interest to determine if other obligate dimers like Gβγ are also co-translationally assembled.

CHAPTER 3

ROLE OF PHLP2 AND PHLP3 IN ACTIN AND TUBULIN FOLDING IN MAMMALIAN CELLS

Summary

PhLP2 and PhLP3 are members of the phosducin gene family that are known to interact with the cytosolic chaperonin complex (CCT). In so doing, they may regulate CCT-mediated actin and tubulin folding. Their functional role in CCT-mediated actin and tubulin folding is different *in vitro* and *in vivo*. While *in vitro* experiments suggest a negative regulatory role, *in vivo* studies in yeast suggest otherwise. The results of this study show that PhLP2A is not involved in actin, α-tubulin and β-tubulin folding, while PhLP3 may contribute to actin folding, but not α-tubulin or β-tubulin folding. These results indicate very different roles for PhLP2A and PhLP3 in human cells compared to their proposed roles in yeast.

Introduction

To reach their native three dimensional state, significant numbers of proteins rely on a group of proteins called chaperones (Hartl and Hayer-Hartl, 2002). An intriguing class of oligomeric, high-molecular-weight chaperones with the unique ability to fold some cytosolic proteins that cannot be folded by simpler chaperone systems are called chaperonins (Frydman, 2001). This unique group of chaperones consists of two-ring assemblies with a central cavity where substrate polypeptides interact and are protected from the cytosolic milieu until they can reach their native state (Bukau and Horwich, 1998; Gutsche et al., 1999). In eukaryotes, CCT

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(chaperone-containing TCP1, also called TRiC, TCP-1 ring complex) was initially proposed to fold only actin and tubulin, but many other substrates have recently been discovered (Dekker et al., 2008; Thulasiraman et al., 1999; Yam et al., 2008), including a class of proteins containing WD 40 repeats, a 40 amino acid repeat ending in a tryptophan-aspartic acid (WD) sequence (Valpuesta et al., 2002). Several proteins including the phosducin-like proteins have been reported to bind mammalian and yeast CCT (Lukov et al., 2005; Martin-Benito et al., 2004; McLaughlin et al., 2002a; Stirling et al., 2006). In so doing, they modulate the folding and ATPase activities of CCT.

In humans three phosducin gene family members form ternary complexes with CCT and it substrates (Willardson and Howlett, 2007). These include PhLP1, PhLP2A, PhLP2B, PhLP3, while the original member of the gene family phosducin does not bind CCT (Willardson and Howlett, 2007). Of the two PhLP2 genes in humans and mice, PhLP2A is a ubiquitously expressed phosphoprotein (Wilkinson et al., 2004), while PhLP2B has been reported to only be expressed in male and female germ cells undergoing meiotic maturation (Lopez et al., 2003). Just like PhLP1, PhLP2A and PhLP3 have been shown to inhibit CCT-actin and CCT-tubulin folding in *in vitro* translation systems (Stirling et al., 2006; Stirling et al., 2007). The ATPase activity of CCT in actin and tubulin folding is also inhibited by PhLP3 (Stirling et al., 2006; Stirling et al., 2007). However, *in vitro* and *in vivo* experiments to elucidate the function of PhLP2A suggest that it inhibits CCT-actin folding while its yeast orthologue PLP2 positively regulates CCT and actin function (Stirling et al., 2007). This positive regulatory role of yeast PLP2 was confirmed by an *in vitro* yeast CCT-

ACT1 folding assay in which PLP2-CCT-ACT1 complexes yielded 30-fold more native actin than CCT-ACT1 complexes (McCormack et al., 2009). Like PhLP2, there are also discrepancies in the function of PhLP3 in the folding of cytoskeletal proteins actin and tubulin. While *in vivo* genetic analysis in yeast suggest a positive role of PhLP3 in tubulin folding (Lacefield and Solomon, 2003; Ogawa et al., 2004; Stirling et al., 2006), *in vitro* β-tubulin folding assays suggest otherwise (Stirling et al., 2006). Human PhLP3 perturbs actin or β-tubulin folding *in vitro*, while its yeast orthlogue PLP1 appears to coordinate the proper biogenesis of actin and tubulin with prefoldin (Stirling et al., 2006).

The current study was conducted to further investigate the effect of PhLP2A and PhLP3 in actin and tubulin folding in mammalian cells. The results show that PhLP2A is not involved in actin, α-tubulin and β-tubulin folding, while PhLP3 may contribute to actin folding, but not α-tubulin or β-tubulin folding. These results indicate very different roles for PhLP2A and PhLP3 in human cells compared to their proposed roles in yeast.

Experimental Procedures

Cell Culture - HEK293T cells were cultured in DMEM/F-12 (50/50 mix) growth media supplemented with L-glutamine, 10% fetal bovine serum and 15 mM HEPES (Hyclone Scientific). In order to maintain active growth, cells were subcultured regularly but not beyond 15 passages.

RNA Interference - Small interfering RNAs (siRNAs) were chemically synthesized (Dharmacon) to target nucleotides 345-365 of human PhLP1 (Lukov et

al., 2005), nucleotides 181-199 of human PhLP2A, nucleotides 989-1007 of human PhLP3 and nucleotides 172-192 of human CCTζ-1 (Grantham et al., 2006). All the oligonucleotides had 3′dTdT overhangs. HEK293T cells were cultured in 12 well plates to 40-65% confluency. Cells were then transfected with the appropriate siRNA at 100 nM final concentration using Oligofectamine (Invitrogen) (Lukov et al., 2005). Cells were harvested for subsequent immunoprecipitation experiments 96 hours later. To assess the percent knockdown of specific proteins, 15 µg of cell lysate were immunoblotted with an anti-PhLP1 antiserum (Thulin et al., 1999) , an anti-CCTζ antibody (Santa Cruz Biotechnology), an anti-PhLP2A antiserum prepared in rabbits against amino acids I_{216}EDVLLSSVRRSVLMKRDSD$_{235}$, and an anti-PhLP3 antibody prepared in rabbits against the full-length PhLP3 protein to determine the extent of knockdown.

Transient Transfections - HEK 293T cells were treated with siRNA and then transfected 24 hrs later with 1.0 µg of C-terminal myc-tagged PhLP1, PhLP2A, or PhLP3; or C-terminal Flag-tagged tubulin co-factor A or tubulin co-factor B as indicated using Lipofectamine Plus Reagent according to the manufacturer's protocol (Invitrogen). The cells were used in subsequent applications after 72 hrs. For over-expression experiments not involving siRNA treatment, HEK 293T were plated in 12 well plates so that they were 70-80% confluent the next day. The cells were then transfected with 1.0 µg cofactor A-Flag, cofactor B-Flag, PhLP1-myc, PhLP2A-myc, 14-3-3ε-Flag, PhLP3-myc and empty vector (pcDNA3.1 (+)) as indicated. The cells were harvested for subsequent applications after 48 hrs.

Radiolabel Pulse-Chase Assay - Vector transiently transfected and siRNA-treated HEK293T cells in 12-well plates were washed and incubated in 1000 µl of methionine free media (Mediatech, Inc) supplemented with 4 mM L-glutamine (Sigma), 0.063g/L L-cystine dihydrochloride (USB) and 10% dialyzed fetal bovine serum (Hyclone). The media was discarded and 400 µl of new media supplemented with 200 µCi/ml radiolabeled L-[^{35}S] methionine (Amersham Biosciences) was added. The cells were then incubated at 23°C for 10 min. to incorporate the [^{35}S] methionine into newly synthesized proteins. After the pulse phase, the cells were washed and incubated at 23°C for 15 min. in 2 ml of media supplemented with 4 mM non-radiolabeled L-methionine and 4 µM cycloheximide (Sigma) to stop the [^{35}S] methionine incorporation. Following the chase period the cells were harvested for immunoprecipitation experiments.

Immunoprecipitation - Pulse-chase labeled HEK 293T cells were washed three times with phosphate-buffered saline (PBS, Fisher) to remove free ^{35}S-methionine (Perkin Elmer) and solubilized in actin IP buffer (20 mM HEPES pH 7.4, 50 mM KCl, 1 mM DTT, 0.2 mM CaCl$_2$, 0.5% NP-40, 4 µM cycloheximide, 1 mM PMSF, 40 mM glucose and 6 µl/ml protease inhibitor cocktail per mL (Sigma)) in the case of actin folding. Cells were solubilized in tubulin IP buffer (PBS, pH 7.4, 0.5% NP-40 (Sigma), 0.6 mM PMSF, 6 µl/ml protease inhibitor cocktail per mL buffer (Sigma)) in the case of tubulin folding.   The lysates were passed through a 25-gauge needle 10 times and centrifuged at 14,000 rpm for 10 min. at 4ºC in an Eppendorf microfuge. The protein concentration for each sample was determined using the BCA Protein Assay reagent (Thermo Scientific) in the case of actin folding or

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Coomassie Plus Protein Assay reagent (Thermo Scientific) in the case of tubulin

folding and equal amounts of protein were used in the subsequent

immunoprecipitations. 160 μg of total protein from the clarified lysates were

incubated for 1 hour at 4°C in the presence of DNase I-affigel beads prepared as

described previously (Rosenblatt et al., 1995) in the case of actin folding, or for 30

min. at 4°C with 2 μg anti-Flag (clone M2, Sigma) followed by an additional 30 min.

at 4°C with 25 μl of a 50% slurry of Protein A/G Plus agarose (Santa Cruz

Biotechnology) in the case of tubulin folding. Samples were centrifuged for 2 min at

4,000 rpm in an Eppendorf microfuge and washed 3 times with 1 ml of IP buffer.

The immunoprecipitated proteins were solubilized in SDS sample buffer and resolved

on a 10% Tris-glycine-SDS gel. The gels were dried on Whatman filter paper for

subsequent radioactivity measurements. Gels were visualized with a Storm 860

phosphor imager and the band intensities were quantified using the Image Quant

software (GE Healthcare).

For 14-3-3 co-immunoprecipitation experiments, HEK-293 cells in 6-well

plates were transfected with 1.0 μg of the indicated PhLP-myc cDNAs along with 1.0

μg of C-terminal Flag tagged 14-3-3ε as described above. The 14-3-3ε was

immunoprecipitated as described above and immunoblotted for the indicated myc-

PhLP isoforms.

Results

To better elucidate the function and physiological role of human PhLP1,

PhLP2A and PhLP3 in CCT-mediated actin and tubulin folding in mammalian cells,

the effects of their siRNA-mediated knockdown on the folding of β-actin, α-tubulin and β-tubulin were measured in HEK-293 cells. The siRNA treatment resulted in 70-80% depletion of the endogenous PhLP1, PhLP2A and PhLP3 (Fig. 3-1 A-C). The effects of this depletion on nascent β-actin folding was measured by pulsing the cells with [^{35}S] methionine and then chasing with unlabeled methionine and cycloheximide, blocking further translation and [^{35}S] incorporation into proteins. After the chase period, the amount of nascent β-actin synthesized and properly folded during the pulse-chase was determine by the binding of the [^{35}S] labeled actin to DNase I immobilized on agarose beads. It has been previously shown that native monomeric β-actin binds to DNase I with high affinity, and this method has been used to measure actin folding in cells (Farr et al., 1997). Interestingly, PhLP1 and PhLP2A depletion had no effect on β-actin folding (Fig. 3-1 E-F) while PhLP3 depletion showed a consistent 40% decrease in actin folding (Fig. 3-1G). As a positive control, the effect of CCTζ knockdown on actin folding was also measured. β-actin folding is very sensitive to CCT depletion given the fact that CCT is required for actin folding (Grantham et al., 2006) . CCTζ knockdown resulted in a 60% depletion of CCTζ (Fig. 3-1 D) and a similar reduction in CCT complexes (Howlett et al., 2009) . Accordingly, a 60% reduction in β-actin folding was also observed, indicating that this method of measuring actin folding was accurate. Thus, the observed decrease in β-actin folding upon PhLP3 depletion suggests a positive regulatory role for PhLP3 in β-actin folding.
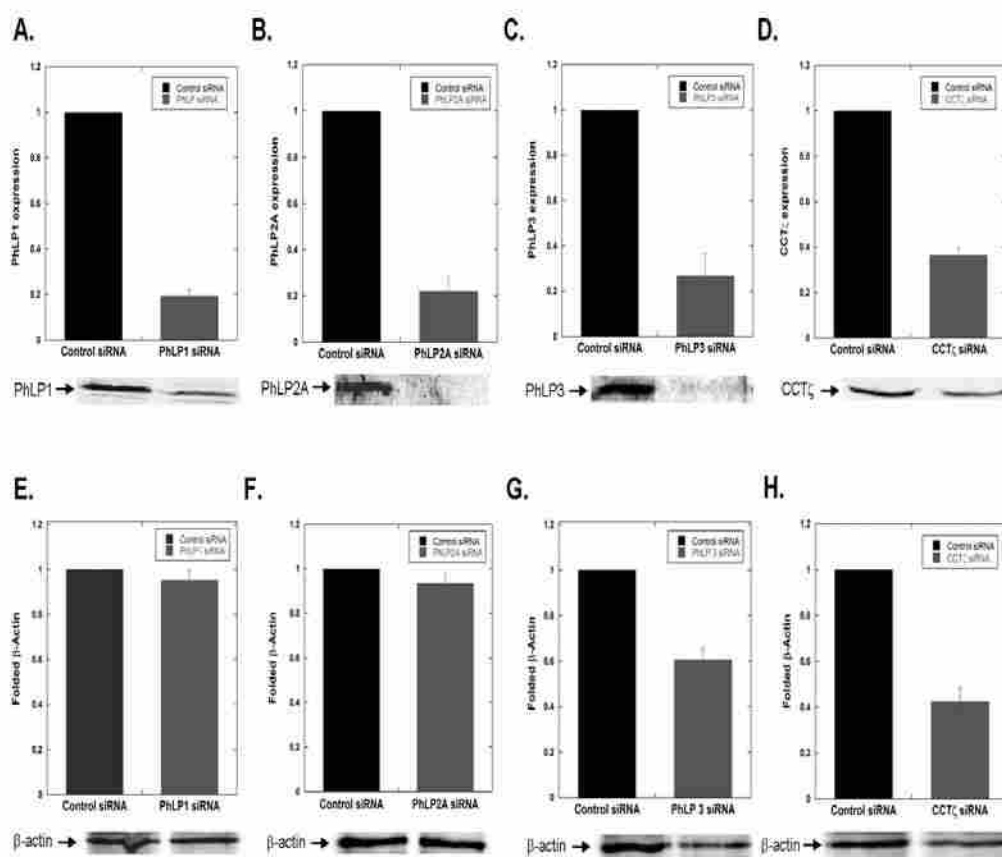
Figure 3-1. Effect of siRNA-mediated knockdown of PhLP isoforms on actin folding.
HEK-293T cells were treated with control siRNA or siRNA specific to PhLP1 (A), PhLP2A (B), PhLP3 (C) or CCTζ (D) as described in Experimental Procedures. Cell extracts were immunoblotted and quantified to determine the efficiency of the siRNA knockdown. The effect of these knockdowns on actin folding was determined by measuring the binding of nascent actin to DNase I beads in an [^{35}S] pulse-chase experimental format as described in Experimental Procedures. In each experiment, the amount of actin bound to the DNase I beads was calculated as a fraction of that bound in the scrambled siRNA control. Bars represent the average ± standard error from at least three experiments. Representative gels are shown below the graphs.

To further assess the effects of PhLPs on actin folding, PhLP1, PhLP2A or

PhLP3 were over-expressed in HEK 293T cells and the effects on β-actin folding

were again measured. Over-expression of each of these PhLPs caused between 40-

60% inhibition of β-actin folding (Fig. 3-2). It was previously reported that the

decrease in actin folding due to PhLP1 was a result of direct competition with binding

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substrates of CCT (McLaughlin et al., 2002a). Moreover, inhibition of β-actin

folding by PhLP2A using a mammalian *in vitro* translation and folding assay has also

been reported (McCormack et al., 2009). It is not clear why both depletion and over-

expression of PhLP3 would inhibit β-actin folding. Perhaps the endogenous levels of

PhLP3 are optimal to assist in β-actin folding and excess PhLP3 somehow interferes

with the folding process (see Discussion).
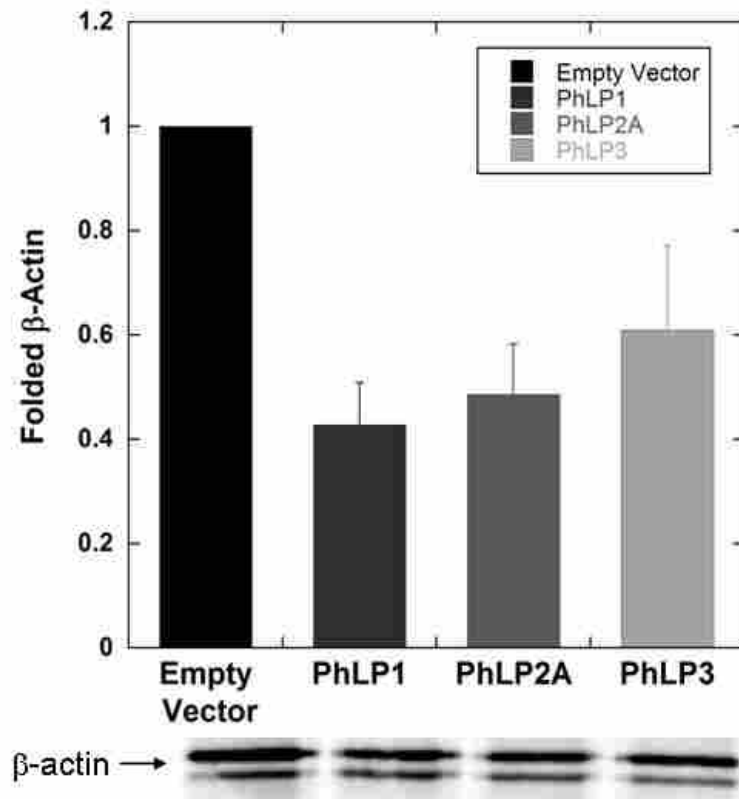
Figure 3-2. Effect of over-expression of PhLP isoforms on actin folding.
HEK-293T cells were transfected with cDNA constructs for PhLP1, PhLP2A, PhLP3 or an empty
vector control along with a Flag β-actin construct as described in Experimental Procedures. The
effect of these over-expressions on actin folding was determined by measuring the binding of the
over-expressed and endogenous actin to DNase I beads in an [^{35}S] pulse-chase experimental format as
in Fig. 3-1. Bars represent the average ± standard error from at least three experiments. A
representative gel is shown below the graph with the upper band corresponding to Flag-tagged β-actin
and the lower band corresponding to endogenous β-actin. Both bands were quantified in the analysis.

A proteomic screen for PhLP2A binding partners had previously suggested an interaction with 14-3-3 proteins (B.M.W. unpublished observations). 14-3-3s are abundant proteins known to bind phospho-serine and phospho-threonine residues and modulate protein function in a phosphorylation-dependent manner (Yaffe, 2002). To confirm this interaction, a co-immunoprecipitation experiment was performed in which each PhLP family member was over-expressed in HEK-293T cells along with 14-3-3ε. After immunoprecipitating the 14-3-3ε, the co-immunoprecipitation of PhLPs was determined by immunoblotting. Only PhLP2A was found in the 14-3-3ε immunoprecipitate (Fig. 3-3 A). The interaction was investigated further by focusing on the C-terminus of PhLP2A, which has two phosphorylation consensus sites for the kinase CK2 at S234 and S236, and phosphorylation of these sites has been shown in global phospho-proteome screens (Dephoure et al., 2008; Kim et al., 2005). A truncation of the C-terminal residues 233-239 resulted in a PhLP2A variant with significantly reduced 14-3-3ε binding (Fig. 3-3 B), indicating that part of the interaction between PhLP2A and 14-3-3ε occurs at the C-terminus. The significance of the PhLP2A/14-3-3ε interaction was further investigated by measuring the effect of 14-3-3ε over-expression on β-actin folding. In the absence of PhLP2A, 14-3-3ε over-expression had no effect on β-actin folding, but in the presence of PhLP2A, 14-3-3ε relieved the inhibition caused by PhLP2A (Fig. 3-3 C). This effect was dependent on the C-terminus of PhLP2A because inhibition of β-actin folding by the PhLP2A Δ233-239 variant was not relieved by 14-3-3ε (Fig. 3-3 C).
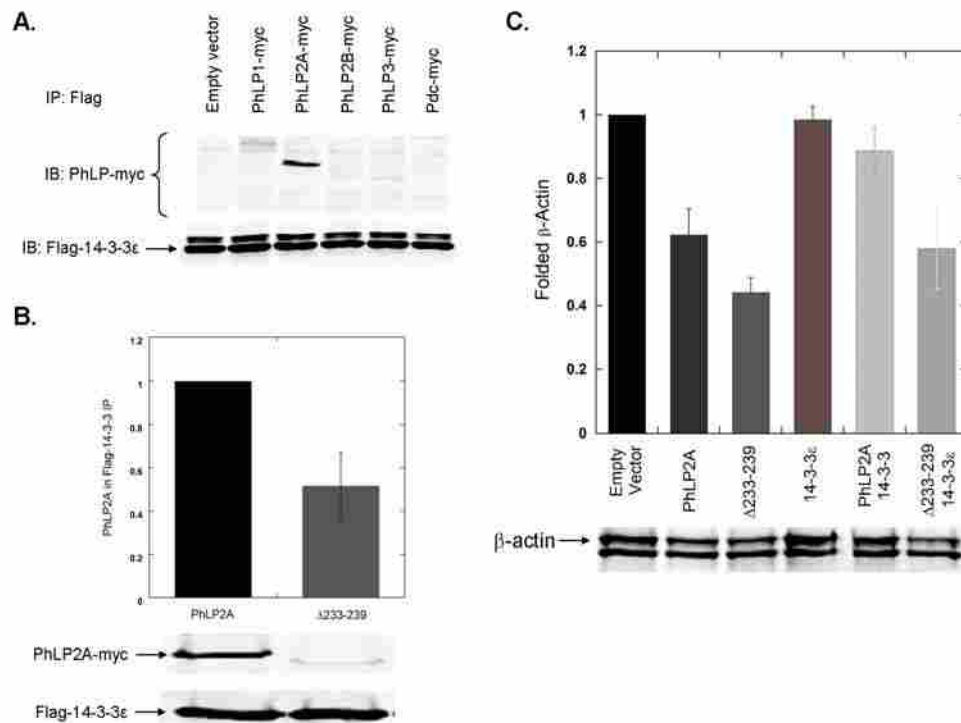
Figure 3-3. Effect of the PhLP2A/14-3-3ε interaction on actin folding.
A) The binding of 14-3-3ε to PhLP isoforms was determined by co-immunoprecipitation. HEK-293T cells were transfected with cDNA constructs for the indicated PhLP isoforms, each with a C-terminal myc epitope tag, along with a C-terminal Flag-epitope tagged 14-3-3ε. The 14-3-3ε was immunoprecipitated with an antibody to the Flag tag and the presence of PhLP isoforms in the co-immunoprecipitate was detected by immunoblotting with an antibody to the myc tag. B) The co-immunoprecipitation of a PhLP2A Δ233-239, a variant missing the last 7 amino acids, was compared to that of full length PhLP2A. Bars represent the average ± standard error from at least 3 experiments. C) The effect of 14-3-3ε on the inhibition of β-actin folding by PhLP2A or the PhLP2A Δ233-239 variant is shown. β-actin folding was measured as in Fig. 3-2. Bars represent the average ± standard error from at least three experiments. A representative gel is shown below the graph with the upper band corresponding to Flag-tagged β-actin and the lower band corresponding to endogenous β-actin. Both bands were quantified in the analysis. Panels A and B were from work done by Amy Gray.

These results are consistent with the finding that replacement of the C-terminus of

PhLP2A with the highly divergent sequence from the C-terminus of PLP2 from yeast

also relieved the inhibition of β-actin folding in rabbit reticulocyte lysates

(McCormack et al., 2009). These results suggest that PhLP2A may be regulating β-

actin folding in a 14-3-3ε dependent manner.

It has been hypothesized that different PhLPs favor the folding of different

CCT substrates (Willardson and Howlett, 2007). To further test this notion, the

effects of PhLP1, PhLP2A and PhLP3 on the folding of two additional important

CCT substrates, α- and β-tubulin, were also determined. To perform these

experiments in cells, new tubulin folding assays were developed. Previous assays

measured the incorporation of nascent α- and β-tubulin into microtubules (Yaffe et

al., 1992) . This assay was cumbersome and not sufficiently sensitive for the small

scale cell culture format necessitated by siRNA knockdown methods. Therefore a

new assay was developed which takes advantage of the binding of α-tubulin to

tubulin co-factor B upon release from CCT and the binding of β-tubulin to tubulin co-

factor A upon release from CCT. These co-factors assist in the formation of the αβ

tubulin dimer after the tubulin protomers are folded by CCT (Lopez-Fanarraga et al.,

2001) . These assays followed a similar work flow as the β-actin folding assay,

except that after the pulse-chase period, nascent folded α-tubulin was isolated by co-

immunoprecipitation with over-expressed Flag-tagged co-factor B and nascent folded

β-tubulin was isolated by co-immunoprecipitation with over-expressed Flag-tagged

co-factor A. In the case of α-tubulin, siRNA depletion or over-expression of PhLP1,

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PhLP2A or PhLP3 in HEK-293T cells had no effect on α-tubulin folding (Fig.3- 4),

indicating that α-tubulin folding is independent of these PhLPs in HEK-293T cells. In

the case with β-tubulin, siRNA-mediated depletion of PhLP2A or PhLP3 seemed to

increase β-tubulin folding, while over-expression of PhLP1, PhLP2A or PhLP3 had

little effect.

Figure 3-4. Effect of PhLP isoforms on α-tubulin folding.
 A) HEK-293T cells were treated with siRNA specific to PhLP1, PhLP2A, PhLP3 or a scrambled
siRNA control as indicated. After 24 hours, the cells were transfected with an N-terminal Flag
tagged co-factor B. After 72 additional hours, the binding of nascent α-tubulin to co-factor B was
determined as a measure of α-tubulin folding in a pulse-chase experimental format as described in
Experimental Procedures. In each experiment, the amount of nascent α-tubulin bound to co-factor
B was calculated as a fraction of that bound in the scrambled siRNA control. Bars represent the
average ± standard error from at least three experiments. A representative gel is shown below the
blot. B) HEK-293T cells were transfected with the indicated PhLP cDNA constructs and after 48
hours, α-tubulin folding was measured as described in panel A. In each experiment, the amount of
nascent α-tubulin bound to co-factor B was calculated as a fraction of that bound in the empty
vector control. Bars represent the average ± standard error from at least three experiments. A
representative gel is shown below the blot.

However, the β-tubulin folding results are suspect because the binding of β-tubulin

to co-factor A was weak, resulting in faint β-tubulin bands in the co-

immunoprecipitate that were difficult to quantify and more importantly because

CCTζ depletion did not inhibit β-tubulin folding as would be expected. Thus, it

appears that a different assay will need to be developed to measure β-tubulin folding
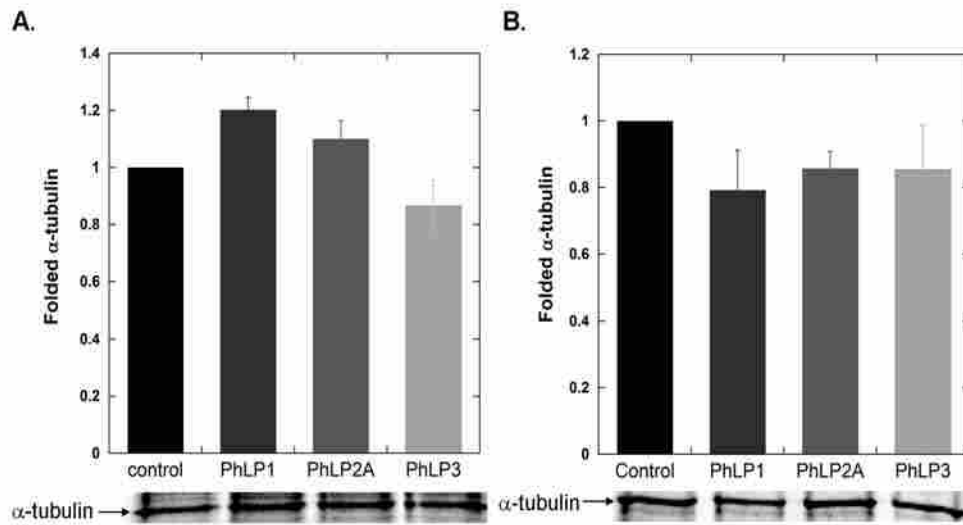
accurately.
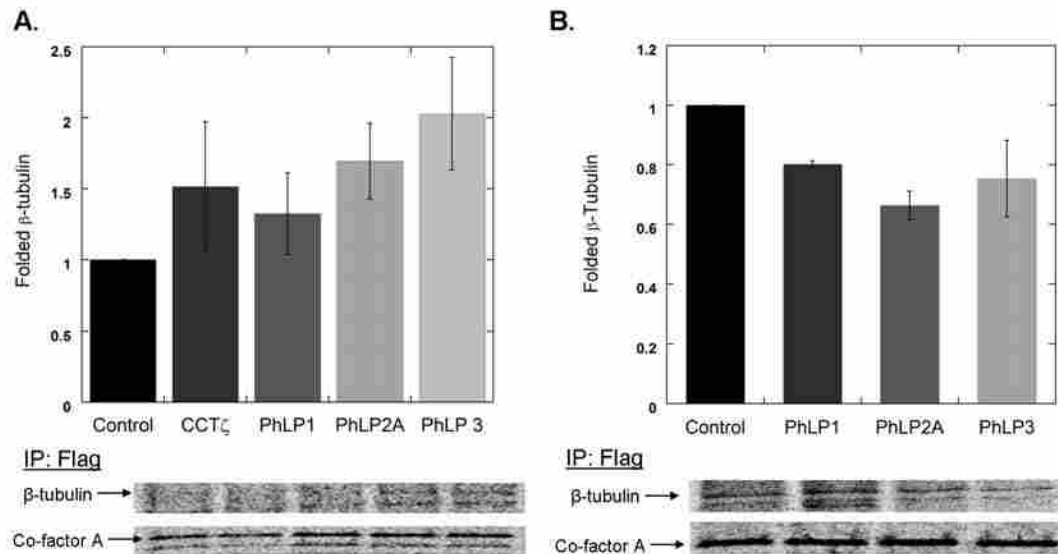
Figure 3-5. Effect of PhLP isoforms on β-tubulin folding.
A) HEK-293T cells were treated with siRNA specific to CCTζ, PhLP1, PhLP2A, PhLP3 or a scrambled siRNA control as indicated. After 24 hours, the cells were transfected with an N-terminal Flag tagged co-factor A. After 72 additional hours, the binding of nascent β-tubulin to co-factor A was determined as a measure of β-tubulin folding in a pulse-chase experimental format as described in Experimental Procedures. In each experiment, the amount of nascent β-tubulin bound to co-factor A was calculated as a fraction of that bound in the scrambled siRNA control. Bars represent the average ± standard error from at least three experiments. Representative gels are shown below the blot. B) HEK-293T cells were transfected with the indicated PhLP cDNA constructs and after 48 hours, β-tubulin folding was measured as described in panel A. In each experiment, the amount of nascent β-tubulin bound to co-factor A was calculated as a fraction of that bound in the empty vector control. Bars represent the average ± standard error from at least three experiments. Representative gels are shown below the blot. This Figure is from work done by Amy Gray.

Discussion

Recent work has shown that all members of the Pdc gene family, except for Pdc itself, form complexes with CCT and in so doing serve as modulators of CCT-mediated protein folding. The key role of PhLP1 as a co-chaperone in the folding of Gβ and in the assembly of the Gβγ dimer has been recently characterized (Lukov et al., 2006; Lukov et al., 2005; Martin-Benito et al., 2004; McLaughlin et al., 2002a). The roles of PhLP2 and PhLP3 in CCT-dependent protein folding are less clear. Genetic studies of PhLP3 in *S. cerevisiae* and *C. elegans* suggest that PhLP3 participates in β-actin and β-tubulin biogenesis (Lacefield and Solomon, 2003; Ogawa et al., 2004; Stirling et al., 2006). PhLP2A is essential for viability in *S. cerevisiae* (Flanary et al., 2000), possibly as a co-chaperone in the folding of cytoskeletal components such as β-actin and β-tubulin or essential cell cycle components (McCormack et al., 2009; Stirling et al., 2006; Stirling et al., 2007). While these reports have begun to define the physiological role of PhLP2 and PhLP3, much is still unknown. In particular, there appears to be significant differences in PhLP function between lower and higher eukaryotes. For example, the yeast orthologue of PhLP2A, PLP2, greatly enhances β-actin folding (McCormack et al., 2009), while mammalian PhLP2A inhibits it, both in rabbit reticulocyte lysate *in vitro* translation and folding assays (McCormack et al., 2009) and in HEK-293 cells (Fig. 3-2). Moreover, the yeast orthologue of PhLP3, PLP1, appears to enhance β-tubulin folding and inhibit β-actin folding (Stirling et al., 2006), while the results reported here suggest that in HEK-293T cells, endogenous PhLP3 enhances β-actin folding while having little or no effect on α- or β-tubulin folding (Figs. 3-1, 3-4 and 3-5).

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It is interesting to note that depletion and over-expression of PhLP3 both

inhibited β-actin folding, though it is not clear why this is the case. It appears that the

endogenous level of PhLP3 in HEK-293 cells is carefully balanced for optimal β-

actin folding. A possible explanation of this phenomenon comes from *in vitro*

experiments in which PhLP3 formed a ternary complex with β-actin and CCT and

inhibited β-actin folding. Perhaps the order of association of β-actin and PhLP3 with

CCT is important in β-actin folding. If β-actin binds CCT first followed by PhLP3,

then efficient folding of β-actin occurs. Such would be the case at endogenous levels

of PhLP3 in HEK-293T cells where β-actin is in large excess. However, when

PhLP3 is over-expressed, it may begin to bind CCT before β-actin and disrupt the

productive binding of β-actin to CCT needed for folding. A similar mechanism may

be responsible for the inhibition of β-actin folding upon over-expression of PhLP1

and PhLP2A. These PhLPs may also block productive β-actin binding to CCT. The

difference between the effects of PhLP1 and PhLP2A compared to PhLP3 may be

that the former cannot form productive ternary complexes with β-actin and CCT no

matter the order of binding.

An alternative explanation for the dual effects of PhLP3 on β-actin folding

may lie in the fact that PhLP3 has been shown to inhibit the ATPase activity of CCT

(Stirling et al., 2006). This activity is essential for β-actin release from CCT.

Perhaps excess PhLP3 slows the release of β-actin from CCT and thereby decreases

the rate of β-actin folding. Whatever the mechanism, it appears that PhLP3 does play

a role in β-actin folding in HEK-293T cells and further studies will be needed to determine precisely what that role is.

The ability of 14-3-3 to relieve the inhibition of β-actin folding by PhLP2A is noteworthy. It has been shown previously that this inhibition is dependent on the C-terminal residues of PhLP2A (McCormack et al., 2009). The results presented here show that 14-3-3 relieves the inhibition of β-actin folding by binding to the C-terminus of PhLP2A. Binding of 14-3-3ε to PhLP2A has been shown to depend on phosphorylation of S234 and S236 in this C-terminal region (Amy Gray unpublished results). These findings add another layer of complexity to the regulation of CCT-mediated protein folding by PhLPs. Not only do certain PhLPs favor the folding of certain substrates and inhibit the folding of others, but it appears that this substrate selection can be regulated by phosphorylation and 14-3-3 binding.

The apparent lack of effect of PhLPs on tubulin folding in HEK-293T cells is surprising, given the evidence for a role of PhLP2A and PhLP3 in tubulin folding in other organisms (Lacefield and Solomon, 2003; Ogawa et al., 2004; Stirling et al., 2006). However, these results need to be verified in tubulin folding assays that are sensitive to CCT knockdown. The binding of nascent α-tubulin to co-factor B was strong and clearly quantifiable bands were observed, but the effects of CCTζ knockdown were not measured. In contrast, the binding of nascent β-tubulin to co-factor A was weak and the bands were not easily quantifiable. Moreover, CCTζ knockdown had little effect on the amount folded in this assay. Therefore, it is unlikely that the binding of nascent β-tubulin to co-factor A is a good measure of β-tubulin folding. Future experiments will address these issues. Perhaps the best

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solution is to measure the amount of nascent β-tubulin bound to α-tubulin in a similar co-immunoprecipitation format. Whatever alternative assays are developed, they should show a decrease in β-tubulin folding upon CCTζ knockdown.

In summary, it appear that PhLP3 may assist in β-actin folding in mammalian cells and that PhLP1 and PhLP2A inhibit actin folding in favor of other CCT substrates. In the case of PhLP1, Gβ is its principal substrate, but the major substrates for PhLP2A have yet to be identified. Understanding the effects of PhLPs on tubulin folding in mammalian cells awaits the development and validation of better folding assays.

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