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Function of phosducin-like proteins in G protein signaling and chaperone-assisted protein folding

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Abstract

Members of the phosducin gene family were initially proposed to act as down-regulators of G protein signaling by binding G protein $\beta\gamma$ dimers ($G\beta\gamma$) and inhibiting their ability to interact with G protein α subunits ($G\alpha$) and effectors. However, recent findings have overturned this hypothesis by showing that most members of the phosducin family act as cochaperones with the cytosolic chaperonin complex (CCT) to assist in the folding of a variety of proteins from their nascent polypeptides. In fact rather than inhibiting G protein pathways, phosducin-like protein 1 (PhLP1) has been shown to be essential for G protein signaling by catalyzing the folding and assembly of the $G\beta\gamma$ dimer. PhLP2 and PhLP3 have no role in G protein signaling, but they appear to assist in the folding of proteins essential in regulating cell cycle progression as well as actin and tubulin. Phosducin itself is the only family member that does not participate with CCT in protein folding, but it is believed to have a specific role in visual signal transduction to chaperone $G\beta\gamma$ subunits as they translocate to and from the outer and inner segments of photoreceptor cells during light-adaptation.

Keywords

G protein signaling; phosducin-like proteins; protein folding; cytosolic chaperonin complex

1. Introduction

Eukaryotic cells use G protein signaling systems to mediate a wide array of hormonal, neuronal and sensory signals that control numerous physiological processes ranging from cardiac rhythm [1] to psychological behavior [2] to vision [3]. The importance of G protein signaling to cellular physiology is evidenced by the large number of genes encoding GPCRs (~ 800 in humans [4]) and the myriad of diseases linked to malfunctions in G protein signaling [5]. In fact, more than half of all currently prescribed pharmaceuticals target GPCRs and other G protein pathway components [6]. Consequently, the mechanisms by which G protein signals are propagated has been described in molecular detail [7]. Signaling is initiated by the binding of a ligand to the extracellular face of a GPCR, resulting in a change in the packing of the seven transmembrane α -helices found in all GPCRs. This conformational change activates the G protein on the intracellular surface of the receptor by initiating an exchange of GDP for GTP on the G protein α subunit ($G\alpha$). GTP binding causes $G\alpha$ to dissociate from the G protein $\beta\gamma$ subunit complex ($G\beta\gamma$). Both $G\alpha$ -GTP and $G\beta\gamma$ control the activity of effector enzymes such as adenylyl cyclase, cGMP phosphodiesterase, phospholipase C β , phosphatidylinositol-3-kinase and Rho guanine

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nucleotide exchange factors as well as K^+ and Ca^{2+} ion channels. These effectors regulate the intracellular concentration of second messengers (cyclic nucleotides, inositol phosphates and Ca^{2+}), the actin cytoskeleton (via Rho-GTP) and the plasma membrane electrical potential (via K^+ channels), thereby orchestrating the cellular response to the stimulus.

Controlling such responses is vital to the cell. Hence, G protein pathways are exquisitely regulated and regulatory targets are found at multiple levels within the cascade. At the level of the GPCR, the ability of agonist-bound receptors to activate G proteins is blocked by phosphorylation, arrestin binding and internalization [2]. Interestingly, this deactivation step with respect to the G protein branch of activation from GPCRs results in initiation of a β -arrestin branch that leads to activation of mitogen-activated protein kinase (MAPK) cascades [8]. At the level of $G\alpha$, the lifetime of many $G\alpha$ -GTP isoforms is decreased by acceleration of GTP hydrolysis by regulators of G protein signaling (RGS) proteins and certain effectors such as phospholipase C β [9]. These GTPase accelerating proteins (GAPs) play a vital role in determining the lifetime of the G protein signal [10]. At the level of $G\beta\gamma$, the binding of Pdc [11-14] and PhLP1 [15,16] to $G\beta\gamma$ has been proposed to control the amount of $G\beta\gamma$ available for interaction with effectors or with $G\alpha$ -GDP. However, this $G\beta\gamma$ sequestration model for Pdc and PhLP1 function has been brought into serious question by recent findings [17-19]. This article will focus on current understanding of the roles of Pdc and PhLP1 in $G\beta\gamma$ signaling and on the other possible functions of Pdc family members.

The Pdc gene family appears to have ancient origins in that its members are widely-expressed in organisms varying from yeast to plants to man. The family can be divided into three subgroups [20]. Subgroup I includes the initial members of the family, Pdc and PhLP1, which have been shown to bind $G\beta\gamma$ subunits with high affinity [11,21,22]. Pdc expression is very restricted, being found at significant levels in only the photoreceptor cells of the retina and in the pineal gland [11,23]. This expression pattern suggests a specific role for Pdc in light signaling. In contrast, PhLP1 is broadly expressed in most tissues and cell types [24,25], indicating a more general function. Subgroup II consists of two recently discovered genes in humans, identified as PhLP2A and PhLP2B [20,26,27]. The yeast homolog of PhLP2 lacks $G\beta\gamma$ binding ability, but is essential for cell growth in both the yeast *Saccharomyces cerevisiae* [28] and the soil amoebae *Dictyostelium discoideum* [20], indicating a vital function that is independent of G protein signaling. Subgroup III consists of a single gene, designated PhLP3 [20]. Again, the yeast ortholog of PhLP3 binds $G\beta\gamma$ poorly [28], but its genetic deletion has no obvious phenotype in yeast [28] or *Dictyostelium* [20]. Closer analyses suggest that PhLP3 may participate β -tubulin and possibly actin folding [29,30]. Together, these data portray the Pdc gene family as one with just a few members whose physiological roles are very diverse, having apparently no single unifying cellular function. However, a common theme appears to be emerging from recent findings which indicate that PhLPs 1-3 may all act as co-chaperones in protein folding while Pdc may have a unique role in $G\beta\gamma$ signaling in photoreceptor cells.

2. The role of phosducin in the rod photoreceptor cells of the retina

2.1. Early observations – the $G\beta\gamma$ sequestration hypothesis

Pdc was first discovered as a retinal phospho-protein that was phosphorylated in the dark by cAMP-dependent protein kinase (PKA) and subsequently dephosphorylated upon exposure to light [31]. Interest in Pdc increased significantly when it was shown to co-purify from retinal extracts with the $\beta\gamma$ subunit complex of the retinal G protein, transducin ($G_t\beta\gamma$) [11]. These observations resulted in the protein being given the name phosducin. A potential role for Pdc in down-regulating G protein signaling was proposed when in vitro experiments showed that phosducin could block G protein signaling by disrupting the interaction between G protein α subunits ($G\alpha$) and $G\beta\gamma$ [12,13]. This disruption resulted in Pdc-mediated inhibition of G_t

binding to its GPCR, light-activated rhodopsin [14]. Moreover, its ability to inhibit G protein signaling was reversed when Pdc was phosphorylated by PKA [13,14]. These findings led to the hypothesis that Pdc could be involved in light-adaptation in photoreceptor cells through a feedback inhibition cycle of light-dependent phosphorylation events [32]. The logic of this hypothesis was as follows. In the dark when cAMP levels in photoreceptors are high [33,34], Pdc is phosphorylated and thus would not compete with $G_t\alpha$ for $G_t\beta\gamma$ binding. In the light, cAMP levels in photoreceptors fall [33,34], resulting in Pdc dephosphorylation and disruption of the binding of $G_t\alpha$ to $G_t\beta\gamma$. This $G_t\beta\gamma$ sequestration hypothesis has influenced thinking about the function of Pdc in the retina for some time and additional evidence has accumulated over time in support of its premises. First, for the sequestration model to be valid Pdc expression levels in photoreceptors would need to equal the very high expression level of transducin found in these cells, otherwise there would not be sufficient Pdc to sequester $G_t\beta\gamma$. This was found to be the case, both G_t and Pdc are expressed at ~ 1 copy per 10 rhodopsins [19,35]. Second, the structure of the Pdc- $G_t\beta\gamma$ complex, solved by X-ray crystallography, was consistent with a $G_t\beta\gamma$ sequestration function for Pdc [36,37]. The structure showed that Pdc bound the same face of $G_t\beta\gamma$ as $G_t\alpha$, with a great degree of overlap between the interaction surfaces. This face of $G_t\beta\gamma$ also constitutes a major interaction site for other $G_t\beta\gamma$ effectors, including G protein receptor kinase 2 (GRK2), phospholipase C β (PLC β), adenylyl cyclase 2 (AC2), GIRK K⁺ channels and N-type Ca²⁺ channels [38]. In addition to the effector binding surface, Pdc also occluded the membrane binding surface of $G_t\beta\gamma$ and buried the C-terminal farnesyl group of $G_t\gamma$ in a cleft of $G_t\beta$ created by Pdc binding [36,37,39]. These observations were consistent with the finding that Pdc formed a soluble complex with $G_t\beta\gamma$ by blocking its binding to rod disc membranes [14]. Third, phosphorylation of Pdc at the major PKA site (serine 73 (S73) in most mammals, serine 71 in the mouse) resulted in destabilization of helix 2 of Pdc, which uncovered residues of $G_t\beta\gamma$ involved in the $G_t\alpha$ interaction that might allow $G_t\alpha$ to displace Pdc from $G_t\beta\gamma$ [40]. This later finding provided a possible mechanism by which Pdc phosphorylation could regulate the interaction between $G_t\beta\gamma$ and $G_t\alpha$. Fourth, evidence was accumulating for a similar role for Pdc in other G protein signaling systems. It was found that addition of Pdc in vitro or over-expression of Pdc in cultured cells resulted in inhibition of $G_t\beta\gamma$ -mediated GRK2 [41], GRK3 [42], PLC β [43] and AC2 [44] activity. These results suggested that Pdc might be a general G-protein regulator through its ability to sequester $G_t\beta\gamma$.

2.2. Modifying the model – new clues about Pdc function

A great deal has been learned in the past decade that has significantly changed our thinking about the function of Pdc. First, the localization of Pdc and $G_t\beta\gamma$ in photoreceptors has been measured more carefully, both by immunohistochemistry [45,46] and by microtome sectioning [19]. These studies have shown that Pdc localization does not mimic that of $G_t\beta\gamma$. Pdc is found throughout the rod photoreceptor cell, from the outer segment to the inner segment and synaptic terminus [19,45,46]. Moreover, its distribution doesn't change in response to light. This leaves $\sim 15\%$ of the total Pdc in the outer segment in both the light and dark [19,45,46]. In contrast, $\sim 70\%$ of $G_t\beta\gamma$ is found in the outer segment in dark-adapted rods, associated with the disc membranes, and $G_t\beta\gamma$ redistributes like a soluble protein to the inner segment and synaptic region in response to intense light that saturates rod responses, leaving only $\sim 15\%$ of $G_t\beta\gamma$ in the outer segment under such conditions [19]. Thus, in the low light regimes in which rods function, there appears to be insufficient Pdc in the outer segments to sequester much $G_t\beta\gamma$. This prediction was confirmed by measuring responses to light flashes in dark-adapted Pdc knockout mice compared to wild-type mice. The ERG a-waves were indistinguishable from those of wild-type mice [19], indicating that Pdc had no effect on rod responsiveness in the dark-adapted state. There is however enough Pdc to sequester all the $G_t\beta\gamma$ that remains after extended exposure to bright light. Together these data show that Pdc does not regulate normal responses of rods to dim light, but that Pdc could assist in the important process of shutting down rod responsiveness during prolonged exposure to more intense light.

Significant progress has also been made regarding phosphorylation of Pdc and its effect on Pdc function. The original sequestration hypothesis predicted that Pdc phosphorylation by PKA would block its binding to $G_t\beta\gamma$. However, careful measurements of Pdc binding to $G_t\beta\gamma$ showed that PKA phosphorylation only decreased the binding by 3-fold [47,48]. In fact, the complex between PKA phosphorylated Pdc and $G_t\beta\gamma$ was crystallized and its structure determined as mentioned above [40]. These findings raised questions about whether Pdc and $G_t\beta\gamma$ actually dissociated upon PKA phosphorylation in the dark. The answer to this question came when it was discovered that Pdc could also be phosphorylated by a Ca^{2+} /calmodulin dependent kinase (CaMK) and that this phosphorylation disrupted Pdc binding to $G_t\beta\gamma$ 100-fold more than phosphorylation by PKA [47]. Subsequent experiments performed in intact retinas showed that Pdc did indeed bind $G_t\beta\gamma$ much more in the light than in the dark [19,49] and that serine 54 (S54) was the site of CaMK phosphorylation on Pdc that was crucial for disrupting $G_t\beta\gamma$ binding in the dark [49]. Interestingly, this study found that when S73 was phosphorylated but S54 was not, Pdc still bound $G_t\beta\gamma$. In contrast, when both S54 and S73 were phosphorylated, Pdc binding to $G_t\beta\gamma$ was blocked [49]. This result was somewhat puzzling because when both S54 and S73 were phosphorylated in vitro, there was no inhibition of Pdc binding to $G_t\beta\gamma$ [47]. This inconsistency between the results in vitro and in intact retina could be reconciled by the binding of 14-3-3 proteins. Pdc binds 14-3-3 proteins [46,47], but only when both S54 and S73 are phosphorylated [47]. Moreover, 14-3-3 competes with $G_t\beta\gamma$ for binding to Pdc [47]. Thus, it appears that in order to block Pdc binding to $G_t\beta\gamma$ in vivo, both S73 and S54 must be phosphorylated and 14-3-3 proteins must be bound. These results indicate that the light-induced decrease in both cAMP and Ca^{2+} in rods are required for Pdc dephosphorylation and $G_t\beta\gamma$ binding.

A change in our perception of Pdc function in other tissues has also occurred as a result of measurements of Pdc expression levels. Pdc expression approaches $G\beta\gamma$ expression only in the retina and pineal gland, while in other tissues its expression levels are vanishingly low [50]. Thus, there appears to be insufficient Pdc in most cells to sequester enough $G\beta\gamma$ to impact G protein signaling significantly. As a result, Pdc probably does not act as a general $G\beta\gamma$ regulator as initially suggested by in vitro and over-expression experiments.

2.3. Current thinking – Pdc as a chaperone of light-dependent G_t translocation

Despite all that is known about Pdc, a clear understanding of its physiological function has remained elusive. However, important clues about its function have come from studies of the light-dependent G_t translocation phenomenon in the Pdc knockout mouse. In these mice, G_t translocation from the outer segment in the light was less efficient. For example, twice as much $G_t\beta\gamma$ remained in the outer segment in Pdc knock-out mice (~ 33 %) compared to wild-type mice (~ 16 %), suggesting that Pdc assists in $G_t\beta\gamma$ movement out of the outer segment in the light [19]. A possible mechanism for this assistance can be formulated from previous biochemical and structural work (see Fig. 1). It has been known for some time that $G_t\alpha$ and $G_t\beta\gamma$ must associate as a $G_t\alpha\beta\gamma$ heterotrimer in order to bind rod outer segment disc membranes effectively [51] as a result of heterogeneous fatty acid acylation of the $G_t\alpha$ N-terminus [52, 53] and farnesylation of the $G_t\gamma$ C-terminus [54]. When Pdc becomes dephosphorylated in the light and binds $G_t\beta\gamma$ that has dissociated from $G_t\alpha$ -GTP and the disc membrane upon light activation, it blocks rebinding of $G_t\beta\gamma$ to $G_t\alpha$ and the disc membrane after GTP hydrolysis [14]. As a result, both $G_t\alpha$ and Pdc- $G_t\beta\gamma$ would become soluble and could redistribute as observed during translocation. This mechanism is also consistent with the effect of knocking out $G_t\alpha$ on $G_t\beta\gamma$ distribution. Without $G_t\alpha$, $G_t\beta\gamma$ is found throughout the rod cell, distributed as a soluble protein in both light and dark [55].

In addition to impaired release from the outer segment, return of G_t from the inner segment to the outer segment in the dark was also inhibited in the Pdc knockout mouse [19]. At first blush,

this does not seem consistent with a role for Pdc in translocation. One might predict that in the absence of Pdc, $G_t\alpha$ and $G_t\beta\gamma$ in the inner segment would rapidly form heterotrimers and transport back to the outer segment. However, if G_t heterotrimers form in the inner segment and associate with membranes that do not traffick to the outer segment, then they might remain trapped in the inner segment. Thus, the role of Pdc in the inner segment may be to keep $G_t\beta\gamma$ and $G_t\alpha$ separated until the appropriate place and time during dark adaptation that would allow the G_t heterotrimer to traffick back to the outer segment (Fig. 1). The trigger for return to the outer segment appears to be S54 phosphorylation of Pdc. This idea is based on the observations that in vivo phosphorylation of S54 blocks the binding of Pdc to $G_t\beta\gamma$ and that the rate of S54 phosphorylation and the rate of translocation to the outer segment are similar, with half-lives in the 90 min. range [49,56]. Moreover, it has been recently reported that S54 phosphorylation is localized in the ellipsoid region of the inner segment near the thin cilium that connects the inner and outer segments, while S73 phosphorylation is found throughout the rod cell [57]. Coupling this striking localization of S54 phosphorylation with its role in the release of $G_t\beta\gamma$ from Pdc suggests that $G_t\beta\gamma$ may only dissociate from Pdc in the ellipsoid. In this manner, Pdc may act to ensure that G_t heterotrimers only form in the proper place in the rod where they will associate with membranes that are directly trafficking to the outer segment. Together, these results seem to choreograph a highly orchestrated dance between Pdc, $G_t\alpha$ and $G_t\beta\gamma$ in which $G_t\beta\gamma$ changes its dancing partner at the appropriate time and place to allow efficient G_t translocation during light and dark adaptation (Fig. 1).

Pdc may have other physiological functions as well. Its ability to inhibit $G_t\beta\gamma$ ubiquitination in an in vitro system suggests that Pdc might protect $G_t\beta\gamma$ from proteasome-mediated degradation [58]. This idea is also supported by the ~ 30 % decrease in $G_t\beta\gamma$ expression in Pdc knockout mice [19]. Another idea that has been forwarded is that Pdc might play a role in the nucleus. A small fraction of the total Pdc appears to be localized in the nucleus [45,59], a Pdc C-terminal fragment has been reported to act as a transcription activator [60] and Pdc binds to the photoreceptor-specific transcription factor CRX in vitro [61]. In addition, Pdc has recently been shown to be SUMOylated and SUMOylation machinery has been shown to associate with the nuclear pore complex that imports proteins into the nucleus [50]. Finally, localization studies indicate that Pdc is more concentrated in the synaptic terminus of rods, perhaps suggesting a role for Pdc in synaptic transmission [45]. These possible functions are not mutually exclusive and suggest that Pdc may have multiple physiological roles in photoreceptors.

3. The physiological function of PhLP1

3.1. Initial hypothesis – PhLP1 as a general inhibitor of G protein signaling

A close homolog of Pdc was discovered in a screen for genes whose expression was induced by ethanol in neuronal cell cultures [24]. This protein displayed 65 % sequence homology to Pdc and was consequently given the name of phosducin-like protein (PhLP) [24]. Pdc and PhLP make up subgroup I of the Pdc gene family [20], and therefore PhLP will be referred to here as PhLP1 to distinguish it from subgroup II and III family members. PhLP1 contains an 11 amino acid sequence corresponding to Helix 1 of Pdc that is perfectly conserved [24]. In Pdc, this sequence of Helix 1 is a major site of interaction with $G_t\beta\gamma$ [36]. Accordingly, PhLP1 was shown to bind $G\beta\gamma$ with similar affinity to Pdc [22] and to block interactions of $G\beta\gamma$ with $G\alpha$ and GRK2 in vitro [21,62]. As with Pdc, over-expression of PhLP1 inhibited G protein signaling [16], but unlike Pdc, PhLP1 displayed a broad expression pattern, being found in significant levels in most tissues [24,25,63]. These findings led to the hypothesis that it was PhLP and not Pdc that was the general down-regulator of G protein signaling through $G\beta\gamma$ sequestration.

Since these initial observations, several inconsistencies with a PhLP1-mediated G $\beta\gamma$ sequestration hypothesis have been observed. First, the expression levels of PhLP1 were significantly less than those of G $\beta\gamma$ [25] and had to be increased to well above endogenous levels to begin to inhibit G protein signaling [16], raising questions about the ability of endogenous PhLP1 to sequester much of the G $\beta\gamma$ pool in the cell. This moderate expression level of PhLP1 is in contrast to the high expression level of Pdc in photoreceptors, which matches that of G $\beta\gamma$ [19,64] and provides sufficient Pdc to bind a large fraction of the G $\beta\gamma$ and exert a major effect on its subcellular localization and signaling. Second, PhLP1 binding to G $\beta\gamma$ was not regulated by phosphorylation [64,65], suggesting that the interaction is more constitutive in nature and less dependent on a phosphorylation-dependent feedback loop like that of Pdc. Third, deletion of the PhLP1 gene in the chestnut blight fungus *Cryphonectria parasitica* [66] and in *Dictyostelium discoideum* [20] yielded the same phenotypes as deletion of the G β gene, the opposite result of that expected if PhLP1 were a negative regulator of G protein signaling. Moreover, G protein signaling in *Dictyostelium* was abolished by the deletion of PhLP1, confirming a requirement for PhLP1 in G protein function [20]. Fourth, anti-sense oligonucleotide-mediated knockdown of PhLP1 in mouse brain prolonged significantly the period of desensitization induced by both acute and chronic exposure to morphine [63], again suggesting that PhLP1 was not an inhibitor of G protein signaling but rather a promoter of both short and long-term responses to agonists. These inconsistencies raised doubts about the sequestration hypothesis and led to the search for other possible functions of PhLP1.

3.2. Over-turning the paradigm – PhLP1 as an essential co-chaperone in G $\beta\gamma$ assembly

Clues about other functions of PhLP1 came from a proteomics screen for PhLP1 binding partners in which a high affinity interaction of PhLP1 with the cytosolic chaperonin complex (CCT) was discovered [67]. This interaction was later confirmed in yeast protein-protein interaction screens [68,69]. Interestingly, Pdc did not share the ability to binding CCT with PhLP [67]. CCT is an essential chaperone of protein folding found in the cytosol of eukaryotic cells [70,71]. It consists of eight different but related subunits of ~ 60 kDa packed together to form a ring structure [72]. Two identical rings stack on top of each other to form the holo-CCT complex of sixteen subunits [72]. Nascent polypeptides and denatured proteins associate in a large cavity formed in the center of each eight-membered ring [72]. Amino acid residues within the ring make contacts with the unfolded protein and decrease the activation energy required to form the three-dimensional structure of the native protein [73]. Each CCT subunit binds ATP and uses the energy of ATP hydrolysis to drive the folding process [74,75]. Actin and tubulin are major cellular proteins that require CCT to fold, but other substrates have been described. In fact, it has been estimated that 10-15% of cellular proteins are assisted in their folding by CCT [76]. Among the known substrates of CCT are G α [77] and several proteins with seven β -propeller WD40 structures similar to that of G β [71,78,79]. PhLP1 did not bind CCT as a folding substrate, but rather it interacted in its native form, suggesting a regulatory role for PhLP1 in CCT-dependent folding [67].

Important insight into the function of the PhLP1-CCT interaction has come from the structure of the complex determined by cryo-electron microscopy (cryo-EM) [80]. Unlike folding substrates such as actin and tubulin which bind CCT within the folding cavity, PhLP1 bound above the cavity, making contacts with only the tips of the apical domains of the CCT subunits. PhLP1 spanned the folding cavity and constricted the apical domains, effectively occluding the cavity. In many respects, this structure is similar to that of CCT bound to prefoldin, a co-chaperone that delivers actin and tubulin to CCT for folding [81]. These substrates occupy the folding cavity while prefoldin sits above the cavity with protrusions into the cavity [81]. Together, these observations suggested that PhLP1 may act as a co-chaperone for the folding of G β subunits by stabilizing an interaction between G β , G γ and CCT until the G $\beta\gamma$ reaches its native state. This idea was consistent with the observation that when PhLP1 was deleted in

Dictyostelium, G β and G γ no longer associated with the plasma membrane, but exhibited a cytosolic localization that would be expected if the subunits did not associate [20].

These findings set the stage for studies that directly measured the role for PhLP1 and CCT in the folding and assembly of G $\beta\gamma$ [17,18,65,82,83]. In one such study, siRNA-mediated depletion of PhLP1 in mammalian cells resulted in a significant decrease in G β_1 expression that led to a corresponding decrease in G protein signaling without affecting G β_1 mRNA levels [18]. Pulse-chase experiments measuring G $\beta_1\gamma_2$ assembly in these cells showed that the rate of assembly decreased by 5-fold when the cells were depleted of 90% of their PhLP1 and increased by 4-fold when PhLP1 was over-expressed [18]. These results demonstrated that the decrease in G β_1 expression and G protein signaling upon PhLP1 depletion was caused by an inability to form G $\beta\gamma$ dimers. Similar data were obtained when the PhLP1 gene was deleted in *Dictyostelium*; cells were completely devoid of G $\beta\gamma$ dimers [82]. CCT was also strongly implicated in the G $\beta\gamma$ assembly process by the observation that nascent G β s bound to CCT in translation assays in vitro [83]. Moreover, addition of G γ subunits significantly decreased G β binding to CCT while increasing G β binding to G γ in an ATP-dependent manner [83]. Together, these observations indicated that PhLP1 and CCT were somehow acting as co-chaperones in the folding and assembly of the G $\beta\gamma$ dimer.

In order to catalyze G $\beta\gamma$ dimer formation, PhLP1 must be phosphorylated by the protein kinase CK2 within a cluster of three consecutive serines at residues 18-20 (S18-20) [18]. Initially, it was reported that PhLP1 was phosphorylated by CK2 within the S18-20 cluster and that an S18-20A alanine substitution variant was more effective at inhibiting G $\beta\gamma$ signaling than wild-type PhLP1 [84]. However, it was unclear why the PhLP1 S18-20A variant was a better inhibitor given that CK2 phosphorylation of PhLP1 did not change its binding affinity for G $\beta\gamma$ [65]. Subsequently, the PhLP1 S18-20A variant was shown to block G $\beta\gamma$ assembly in a striking manner [18]. Over-expression of PhLP1 S18-20A in HEK-293 cells decreased the rate of assembly by 15-fold compared to wild-type PhLP1 and by 4-fold compared to an empty vector control [18]. Thus, not only did PhLP1 S18-20A not support G $\beta\gamma$ dimer formation, it was also able to block the ability of endogenous PhLP1 to catalyze assembly in a dominant negative manner. Measuring the effects of various serine to alanine substitutions within the S18-20 sequence on the rate of G $\beta\gamma$ assembly lead to the conclusion that at least two of the three serines must be phosphorylated in order for PhLP1 to effectively support assembly, with S20 phosphorylation being the most important [65].

A useful tool in understanding PhLP-mediated G $\beta\gamma$ assembly was discovered when an N-terminal 75 amino acids truncation of PhLP1 (PhLP1 Δ 1-75) was found to be an even more effective dominant negative inhibitor than PhLP1 S18-20A [18]. Over-expression of PhLP1 Δ 1-75 completely blocked the G $\beta\gamma$ assembly process in HEK-293 cells [18]. This variant lacks both the S18-20 phosphorylation site as well as the conserved G $\beta\gamma$ binding region corresponding to Helix 1 of Pdc. As a result, PhLP1 Δ 1-75 could not be phosphorylated and bound G $\beta\gamma$ poorly, yet it maintained its full CCT binding capacity [18]. Interestingly, the PhLP1 Δ 1-75 variant is very similar to a naturally occurring PhLP1 truncation (designated PhLP1_s) that is missing the N-terminal 83 amino acids due to alternative mRNA splicing [24,84]. When over-expressed PhLP1_s blocked G β and G γ expression and strongly inhibited G $\beta\gamma$ signaling [17,84], as would be predicted by the effects of PhLP1 Δ 1-75 on G $\beta\gamma$ assembly. It is noteworthy that very little PhLP1_s is found in most tissues, but significant amounts are found in the adrenal gland [25,84]. This expression pattern for PhLP1_s is strikingly similar to the tissue distribution of PhLP1 S18-20 phosphorylation. PhLP1 is completely phosphorylated within the S18-20 site in most tissues, but in the adrenal gland this site is predominantly dephosphorylated [84]. These observations suggest that G $\beta\gamma$ synthesis in the adrenal gland may be down-regulated significantly by these PhLP1 species.

The data presented thus far establish the need for PhLP1 and CCT in G β γ dimer formation, but they give little insight into the mechanism by which this process occurs. The cryo-EM structure of PhLP1-CCT suggested that PhLP1 might stabilize the binding of nascent G β to CCT by forming a ternary complex with G β positioned in the CCT folding cavity and PhLP1 sitting above the cavity [80]. Contrary to this prediction, over-expression of PhLP1 was found not to increase but rather to decrease the binding of G β to CCT [65]. However, over-expression of the PhLP1 S18-20A and Δ 1-75 variants resulted in a large increase in G β binding to CCT, indicating that when PhLP is not phosphorylated, a stable PhLP1-G β -CCT ternary complex is formed [65]. Thus, it appears that PhLP1 phosphorylation destabilizes the ternary complex. This idea is supported by the fact that the rate of release of nascent G β from CCT was accelerated by PhLP1 but was inhibited by PhLP1 S18-20A [65]. The inability of PhLP1 S18-20 and Δ 1-75 variants to release G β from CCT explains their dominant negative effect on G β γ assembly. These variants would compete with endogenous PhLP1 for binding the G β -CCT complex by forming stable ternary complexes that would not release G β .

The mechanism of G β release may involve steric repulsion between the phosphates in the S18-20 cluster and negatively charged residues on the apical domains of CCT. This repulsion would cause the dissociation of a phosphorylated PhLP-G β intermediate that would subsequently associate with G γ . Support for a PhLP-G β intermediate comes from several observations. First, complexes of nascent PhLP1 and G β were found that do not contain G γ [18]. Second, G γ did not accelerate the rate of G β release from CCT beyond that observed in the presence of PhLP1 [65]. Third, G γ did not interact with CCT either directly or in a complex with G β [65,83]. Interestingly, a separate chaperone for G γ has very recently been reported to be DRiP78, an ER membrane protein of the Hsp40 chaperone family that participates in GPCR trafficking [85]. PhLP1 was also shown to interact with DRiP78, suggesting that the PhLP1-G β complex may interact with G γ -bound DRiP78 to facilitate G β γ dimer formation [85]. From these observations, a mechanistic model of G β γ assembly can be proposed as depicted in Fig. 2. PhLP1 plays a central role in this model by releasing nascent G β from CCT in a PhLP1-G β complex that then picks up nascent G γ from DRiP78 to form the G β γ dimer at the ER membrane. Subsequent association of G α with G β γ on the membrane would release PhLP1 for additional rounds of dimer formation.

4. The emerging roles of other PhLP family members

Other members of the Pdc family were first identified in a search for Pdc-like proteins in yeast [28]. They were originally named Plp1 and Plp2, but later phylogenetic analysis placed Plp1 in subgroup III and Plp2 in subgroup II of the Pdc family, so the current convention is to refer to group II subfamily members as PhLP2 and group III members as PhLP3 [20]. There is a high degree of sequence homology between all Pdc family members in the C-terminal ~ 150 amino acids (Fig. 3), indicating that all probably retain the thioredoxin fold of the C-terminal domain of Pdc. In contrast, their N-terminal regions differ significantly [20,28]. The N-terminal domains of Pdc and PhLP1 both contain a conserved 11- amino acid sequence of Helix 1 which is imperative in binding G β γ , while PhLP2 and PhLP3 do not have this sequence and they bind G β γ poorly (Fig. 3) [28]. PhLPs 1-3 all contain an acidic sequence in the loop between Helix 2 and 3 of the Pdc structure that is not well-conserved in Pdc [20]. This acidic region has been shown to play an important role in the binding of PhLP1 to CCT [18,80], and accordingly PhLPs1-3 all bind CCT while Pdc does not [30,67,86]. Apart from this loop and the Helix 3 region that follows, there is very little homology in the N-terminal domain between Pdc subfamily members [20]. PhLP2 and PhLP3 are believed to bind CCT in a manner analogous to PhLP1, as native binding partners and regulators of CCT and not as nascent folding substrates [30,67,86]. These findings suggest that PhLP2 and PhLP3 might function like PhLP1 as co-chaperones with CCT in protein folding.

4.1. PhLP2 – an essential gene involved in CCT-dependent protein folding

Phlp2 genes have been found in many eukaryotic genomes including human, mouse, zebrafish, and fly and have been shown to have an essential function in *Dictyostelium discoideum* and *Saccharomyces cerevisiae* [20,28]. Deletion of the *phlp2* gene in yeast yielded spore products that failed to grow [28], while disruption of PhLP2 in *Dictyostelium* led to a decreasing growth rate and simultaneous collapse of the cell culture after 16-17 cell divisions [20]. The essential function of PhLP2 appears to be separate and unrelated to G β γ signaling as indicated by the lack of effect of PhLP2 and PhLP3 over-expression or PhLP3 deletion on the G β γ -dependent mating pheromone response in yeast [28]. Furthermore, yeast temperature sensitive PhLP2 mutants show no change in sensitivity of their pheromone response at restrictive temperatures [86]. In humans and mice there are two *phlp2* genes designated as *phlp2A* and *phlp2B* [20]. These two genes share 57% sequence homology, but differ in expression patterns [26]. PhLP2A is a ubiquitously-expressed cytosolic phosphoprotein [26], while PhLP2B is only expressed in male and female germ cells undergoing meiotic maturation [27]. As a result of this limited expression in the mouse, PhLP2B was initially referred to as mouse germ cell-specific phosphoducin-like protein (MgcPhLP) [27]. Interestingly, PhLP2B expression is able to rescue the lethal phenotype of yeast *phlp2* Δ , indicating an evolutionarily-conserved function. Given their sequences similarity, shared CCT binding capability and distinct expression patterns, it is believed that PhLP2A and PhLP2B have similar, albeit tissue-specific, functions.

Analyses of temperature sensitive *phlp2* mutants in yeast suggest a possible role for PhLP2 in proper cell cycle progression and cytoskeletal function [86]. A screen to identify genes that partially rescued the lethal defects of *phlp2* mutants at restrictive temperatures revealed several promoters of the G1/S cell cycle transition [86]. In addition, temperature sensitive *phlp2* mutants exhibited a delay in DNA replication and impeded S-phase entry [86]. Interestingly, temperature sensitive mutants of CCT subunits also displayed defects in cell cycle progression [78,79], suggesting a possible co-chaperone role of PhLP2 with CCT in the folding of components essential in regulating cell cycle progression. The temperature sensitive *phlp2* mutants also harbored defects in cytoskeletal function. Growth at semi-permissive temperatures was sensitive to the microfilament disrupting drug latrunculin and to a lesser extent, the microtubule disrupting drug benomyl [86]. Moreover, the mutants displayed significantly larger cell sizes and budding defects, which also suggest actin or tubulin deficiencies [86]. These results point to a role for PhLP2 in the folding of actin and possibly tubulin by CCT. However, in vitro experiments show that while human PhLP2A forms a ternary complex with CCT and actin much like PhLP1, CCT and G β , this complex is inactive and actin folding is inhibited by PhLP2A [86]. This discrepancy between the in vivo phenotypes and the in vitro results could be explained if PhLP2 were not directly required for actin folding, but for the folding of actin-associated proteins necessary for cytoskeletal function. Alternatively, necessary co-factors for PhLP2-mediated actin folding may be missing in vitro. The cytoskeletal defects in *phlp2* mutants are probably not responsible for the observed cell cycle defects because genes that partially rescued the cell cycle phenotype did not affect the cytoskeletal phenotype [86]. Perhaps the best explanation of the *phlp2* temperature sensitive mutant results is that PhLP2 participates with CCT in the folding of several substrates important in cell cycle control and cytoskeletal function [86].

Human PhLP2A has also been referred to as viral inhibitor of apoptosis-associated factor (VIAF) because of an interaction between PhLP2A and the baculovirus *Orgyia pseudotsugata* inhibitor of apoptosis protein (Op-IAP) that was discovered in a human B cell yeast two-hybrid screen [26]. Further investigation proved that PhLP2A does not serve as an antagonist to Op-IAP, but that PhLP2A is ubiquitinated by Op-IAP [26]. However, PhLP2A was shown to play an essential role in the progression of apoptosis when siRNA knockdown of PhLP2A was found to completely inhibit the processing of caspase-3 following the initiation

of Bax-induced programmed cell death [26]. Given the apparent role of PhLP2A in CCT-dependent protein folding, these results may best be explained by PhLP2-assisted folding of one or more proapoptotic factors.

4.2. PhLP3 – a potential co-chaperone for β -tubulin and other CCT substrates

Despite the high degree of homology in their C-terminal domains and their shared ability to bind CCT, PhLP3 has a physiological function distinct from PhLP2. This conclusion stems from the very different phenotypes of PhLP2 and PhLP3 deletions in yeast and *Dictyostelium*. The PhLP2 deletion in both organisms resulted in a loss of viability whereas PhLP3 deletion had no obvious effect [20,28]. Moreover, PhLP3 over-expression did not rescue the lethality of PhLP2 deletion [28]. Further genetic analyses have suggested a role for PhLP3 in β -tubulin folding. In yeast, deletion of PhLP3 protected cells against the toxic effects of excess free β -tubulin, suggesting that PhLP3 is necessary for β -tubulin folding [29,30]. In *C. elegans*, siRNA-mediated knockdown of PhLP3 resulted in defects in microtubule architecture and aberrant cytokinesis, again pointing to a positive role of PhLP3 in tubulin function [87]. Interestingly, cryo-EM studies have demonstrated the formation of a ternary complex between PhLP3, tubulin and CCT, indicating the PhLP3 interacts directly with CCT to regulate β -tubulin folding [30]. In contrast to the positive role of PhLP3 predicted from the genetic studies, in vitro β -tubulin folding assays showed significant inhibition by PhLP3 [30]. This same discrepancy between in vivo genetic phenotypes and in vitro folding measurements was observed with PhLP2 [86]. Again, important co-factors for PhLP3-induced tubulin folding may be missing in the in vitro assays.

PhLP3 also appears to regulate actin function. Genetic deletion of the pac10 subunit of prefoldin in yeast results in a marked decrease in F-actin in the cell, while dual PhLP3 Δ and pac10 Δ deletions restored F-actin to the same level as wild-type [30]. This finding suggests that PhLP3 may somehow down-regulate actin expression or F-actin formation. In support of this finding, PhLP3 inhibits actin folding in in vitro assays [30]. However, the PhLP3 Δ pac10 Δ deletions greatly impaired a number of actin-dependent functions compared to pac10 Δ alone or wild-type cells [30]. These data do not give a clear picture of the role of PhLP3 in actin function possibly as a result of both direct effects on actin folding and indirect effects on actin-associated proteins. Nevertheless, it is clear that PhLP3 does work in concert with prefoldin and CCT to regulate actin function.

5. Conclusion

The initial view of members of the Pdc gene family as down-regulators of G protein signaling through sequestration of G $\beta\gamma$ has been significantly modified in the case of Pdc, completely reversed in the case of PhLP1 and shown to be irrelevant in the case of PhLP2 and 3. It has been replaced by a model in which Pdc family members are more correctly viewed as molecular chaperones. Pdc, the original member of this gene family, has the more specialized function to chaperone G $\beta\gamma$ subunits as they move in and out of the rod photoreceptor in response to light. In contrast, PhLP1-3 all appear to be co-chaperones with CCT in the folding and assembly of different CCT substrates. PhLP1 is an essential component in the assembly of G $\beta\gamma$ dimers, mediating the release of G β from CCT to interact with G γ . PhLP2 is involved with CCT in the folding of yet to be identified cell cycle regulators as well as actin or actin-associated proteins and possibly tubulin. PhLP3 is important in CCT-dependent folding of β -tubulin and possibly actin. In this manner, PhLP isoforms may broaden the range of substrates that are effectively folded by CCT by each assisting a unique subset of substrates in the folding process.

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Fig. 1.

Proposed model of Pdc function in G_t translocation. The diagram summarizes the effects of light on Pdc phosphorylation and the possible role of Pdc in light-dependent $G_t\beta\gamma$ translocation (adapted from [49]). Upon light exposure, Ca^{2+} and cAMP concentrations in rod photoreceptors fall, resulting in dephosphorylation of Pdc at S54 and S73 by PP2A and PP1, respectively. Pdc binds $G_t\beta\gamma$ that has been released from the outer segment disc membranes by light activation and GTP binding to $G_t\alpha$. Pdc maintains $G_t\beta\gamma$ in its soluble form, assisting its diffusion throughout the rod cell. Upon return to the dark, Ca^{2+} and cAMP concentrations rise, and Pdc is phosphorylated at S73 throughout the rod cell, but at S54 only in the ellipsoid region [57]. Upon S54 phosphorylation, 14-3-3 protein binds and $G_t\beta\gamma$ is released from the phosphorylated Pdc. The $G_t\beta\gamma$ then reassociates with $G_t\alpha$ and either binds membranes in the ellipsoid region that are trafficking to the outer segment, or diffuses to the outer segment before

binding to disc membranes there. As dark-adaptation proceeds, other S73-phosphorylated PdcG_tβγ complexes diffuse to the ellipsoid region and are phosphorylated at S54, releasing G_tβγ for G_tα and membrane binding. In this manner, the high concentration of disc membranes in the outer segment acts as a sink to draw G_tβγ into the outer segment, despite the fact that much of the Pdc in the rod cell is only phosphorylated at S73 and is capable of binding G_tβγ (see text for details).

Fig. 2. Proposed model of PhLP1 function in Gβγ dimer assembly. A mechanism describing the role of PhLP1 in Gβγ dimer formation is depicted (adapted from [18]). Nascent Gβ binds CCT within the folding cavity and PhLP1 associates above the Gβ, forming a ternary complex. If PhLP1 is not phosphorylated within the S18-20 sequence by CK2, the ternary complex is stable and inactive in Gβγ assembly. If PhLP1 is phosphorylated at this site, then a PhLP1-Gβ complex is released from CCT and interacts with Gγ bound to DRiP78, forming the Gβγ dimer. PhLP1 is released when Gβγ associates with Gα and the endoplasmic reticulum (ER) membrane, and the G protein heterotrimer is then trafficked to the plasma membrane. PhLP1 is then free to catalyze another round of Gβγ assembly. Note that DRiP78 is shown as a dimer because of the demonstrated dimerization of the J domains of other HSP40 family members [88].

Fig. 3. Sequence alignment of Pdc family members. Sequences of the five human Pdc family members were aligned using CLUSTAL W. Regions of homology between sequences are shaded and gaps are represented by dashes. The structural division between the N-terminal domain and the thioredoxin-like C-terminal domain is indicated by the arrow pointing downward, and the secondary structural components of Pdc are indicated above the sequence (bar – helix, arrow – β -sheet, line – loop, dashed line – unstructured region) [36]. Sites of interaction of PhLP1 with CCT as determined by cyro-EM and mutagenesis analysis are indicated below the sequence (lined bars) [80]. The S18-20 CK2 phosphorylation site of PhLP1 is in bold and underlined as are the S54 CaMK and S73 PKA phosphorylation sites of Pdc.