Role of the Isoprenyl Pocket of the G Protein $\beta\gamma$ Subunit Complex in the Binding of Phosducin and Phosducin-like Protein†

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ABSTRACT: Phosducin (Pdc) and phosducin-like protein (PhLP) regulate G protein-mediated signaling by binding to the $\beta\gamma$ subunit complex of heterotrimeric G proteins (G$\beta\gamma$) and removing the dimer from cell membranes. The binding of Pdc induces a conformational change in the β-propeller structure of G$\beta\gamma$, creating a pocket between blades 6 and 7. It has been proposed that the isoprenyl group of G$\beta\gamma$ inserts into this pocket, stabilizing the Pdc·G$\beta\gamma$ structure and decreasing the affinity of the complex for the lipid bilayer. To test this hypothesis, the binding of Pdc and PhLP to several G$\beta\gamma$ dimers containing variants of the β or γ subunit was measured. These variants included modifications of the isoprenyl group (γ), residues involved in the conformational change (β), and residues lining the proposed prenyl pocket (β). Switching prenyl groups from farnesyl to geranylgeranyl or vice versa had little effect on binding. However, alanine substitution of one residue in the β subunit involved in the conformational change (W332) decreased binding 5-fold. Alanine substitution of certain residues within the prenyl pocket caused only minor decreases in binding, while a lysine substitution of T329 within the pocket inhibited binding 10-fold. Molecular modeling of the binding energy of the Pdc·G$\beta_1\gamma_2$ complex required insertion of the geranylgeranyl group into the prenyl pocket in order to accurately predict the effects of prenyl pocket amino acid substitutions. Finally, a dimer containing a γ subunit with no prenyl group (γ_2-C68S) decreased binding by nearly 20-fold. These results support the structural model in which the prenyl group escapes contact with the aqueous milieu by inserting into the prenyl pocket and stabilizing the Pdc-binding conformation of G$\beta\gamma$.

G protein-mediated signal transduction plays a vital role in cellular signaling processes. Many hormones, neurotransmitters, chemokines, odorants, and even photons of light deliver their signals to cells via G protein-coupled receptors (GPCRs).[1] GPCRs span the plasma membrane of the cell with seven α-helices in a serpentine arrangement. The binding of an agonist at the extracellular face of a GPCR results in a conformational change that creates a high-affinity binding site for a heterotrimeric G protein on the cytoplasmic face of the receptor. This interaction causes the exchange of GDP for GTP on the α subunit of the G protein (Gα), causing a conformational change in Gα that results in dissociation of the complex into three components: Gα·GTP, G$\beta\gamma$, and receptor. The receptor is then free to interact with other G proteins, while Gα·GTP and G$\beta\gamma$ are able to diffuse along the cytoplasmic surface of the membrane and interact with effector enzymes or ion channels and thereby regulate their activity. These effectors in turn control second messenger concentrations (cyclic nucleotides, inositol phosphates, lipids, Ca^{2+}, and K^+) and kinase cascades that dictate the cellular response to the signal (*1*−*3*).

Posttranslational lipid modifications of the Gα and Gγ subunits contribute in important ways to the function of G proteins. Depending on the isoform, Gα subunits can be myristoylated at the N-terminus and/or palmitoylated on a cysteine residue near the N-terminus. These lipid modifications are required for proper plasma membrane localization of Gα, and they regulate the interactions of Gα with G$\beta\gamma$, effectors, and regulators of G protein signaling (RGS) proteins (*4*−*6*). In the case of G$\beta\gamma$, it is prenylated on a cysteine residue in a CaaX motif at the C-terminus of the Gγ subunit with either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid (*7*, *8*). The X residue of the CaaX motif determines which of these two isoprenoids will be linked to Gγ (*9*, *10*). Prenylation of G$\beta\gamma$ is required for membrane association (*11*−*13*), and it affects the interaction of G$\beta\gamma$ with effectors (*14*−*16*) as well as G$\beta\gamma$-dependent coupling of Gα to receptors (*10*, *17*−*19*).

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[1] Abbreviations: GPCR, G protein-coupled receptor; Gα, G protein α subunit; G$\beta\gamma$, G protein $\beta\gamma$ subunit dimer; Pdc, phosducin; PhLP, phosducin-like protein; PLCβ, phospholipase Cβ; AC-II, type II adenylyl cyclase; cAMP, cyclic adenosine monophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris, tris(hydroxymethyl)aminomethane.

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Phosducin (Pdc) is a G$\beta\gamma$-binding protein highly expressed in the retina and pineal (*20*, *21*) and is involved in G protein signaling in these tissues, although its precise role is not known (*22*). Some plausible functions of Pdc in the retina include regulation of the light-dependent movement of the photoreceptor G protein, transducin (G_t), between the outer and inner segments of the photoreceptor (*23*, *24*), protection of G$\beta_1\gamma_1$ (the $\beta\gamma$ subunit combination found in the photoreceptor) from ubiquitination and 26S proteasome degradation (*25*), and regulation of photoreceptor gene transcription (*26*). The interaction of Pdc with G$\beta_1\gamma_1$ causes solubilization of G$\beta_1\gamma_1$ from the plasma membrane, resulting in cytosolic localization of the Pdc·G$\beta_1\gamma_1$ complex (*27*−*29*). Formation of the complex is regulated by phosphorylation. When Pdc is phosphorylated by cAMP-dependent protein kinase and by Ca^{2+}/calmodulin-dependent protein kinase, its interaction with G$\beta_1\gamma_1$ is blocked (*30*−*32*). A homologue of Pdc, termed phosducin-like protein (PhLP), is widely expressed in many tissues (*33*−*35*). Like Pdc, PhLP also binds G$\beta\gamma$, and recent evidence indicates that it is also involved in G protein signaling (*36*−*38*). For example, microinjection of the short splice variant of PhLP into chromaffin cells inhibited catecholamine secretion, a G protein-mediated process (*39*), while overexpression of PhLP blocked G protein signaling through the angiotensin II receptor (*38*) and inhibited G$\beta\gamma$-mediated activation of PLCβ (*40*). In addition, PhLP has also been shown to have other important binding partners, including the cytosolic chaperonin complex (*41*) and the SUG1 subunit of the 26S proteasome (*42*). Thus, it is possible that PhLP acts as a link between G protein signaling and other important metabolic systems.

Determination of the atomic structure of the Pdc·G$\beta_1\gamma_1$ complex in which the C-terminus of the Gγ subunit had been truncated by protease cleavage revealed a conformational change compared to the structure of free G$\beta_1\gamma_1$ and the $G_t\alpha\beta\gamma$ heterotrimer (*43*). The conformational change created a hydrophobic pocket between blades 6 and 7 of the β-propeller [blade numbering follows the convention of Wall et al. (*44*)]. Another structural determination of Pdc·G$\beta_1\gamma_1$ in which the farnesyl-modified C-terminus of Gγ_1 was left intact showed a similar conformational change in G$\beta_1\gamma_1$ (*45*). In addition, this latter structure showed electron density within the pocket created by the conformational change. The electron density fit reasonably well when modeled as the farnesyl group of Gγ_1 tucked into this pocket. However, the resolution of the farnesyl and the last several residues of Gγ_1 were insufficient to definitively assign the prenyl group to this location. Thus, the question of occupation of the pocket by a prenyl group remains open. This Pdc-bound conformation of G$\beta_1\gamma_1$ with the putative prenyl pocket has been referred to as the T (tense) state and the unbound or $G_t\alpha$-bound conformation of G$\beta_1\gamma_1$ has been referred to as the R (relaxed) state (*45*). This nomenclature will be used to refer to the two conformations of G$\beta\gamma$.

The same region of the Gβ subunit that undergoes the conformational change in the T state (the outer strands of blades 6 and 7) has been shown to participate in the interaction of G$\beta\gamma$ with phospholipase Cβ (PLCβ) but not with type I and II adenylyl cyclase (AC-I and -II) (*46*). In addition, changing the prenyl group of G$\beta_1\gamma_2$ from geranylgeranyl to farnesyl decreased the activation of PLCβ by G$\beta_1\gamma_2$ (*14*). Furthermore, substitution of residues involved in the conformational change or residues lining the putative prenyl pocket decreases the ability of G$\beta_1\gamma_2$ to activate PLCβ and AC-II (*47*). From these results, two different ideas have been forwarded for the role of the prenyl group in the interaction of G$\beta\gamma$ with effectors. In the first, the prenyl group of G$\beta\gamma$ interacts directly with effectors, participating in their activation (*15*, *16*). In the second, the T conformation with the prenyl group tucked away constitutes the active form that binds effectors (*14*, *16*, *47*). Determining which of these is the correct role of the prenyl group in G$\beta\gamma$-mediated activation of effectors is complicated by the effects of the prenyl group on membrane association. Since most effectors including PLCβ, AC-I, AC-II, and K^+ channels are membrane proteins, the association of G$\beta\gamma$ with the membrane is necessary for the interaction to occur. If changes in the prenyl group affect membrane binding, then they will also indirectly affect effector binding. In contrast, the interaction of Pdc with G$\beta\gamma$ occurs in the cytosol (*27*), thus removing membrane association effects and making interpretation of the results of prenyl group modifications on Pdc binding simpler.

To investigate the role of the prenyl group of G$\beta\gamma$ on Pdc and PhLP binding, we have measured the interaction of Pdc and PhLP to G$\beta\gamma$ in which the prenyl group was either farnesyl, geranylgeranyl, or completely removed. We have also measured the binding of Pdc and PhLP to G$\beta\gamma$ variants with substitutions in residues involved in the conformational change or residues forming the prenyl pocket. Furthermore, we have calculated the effects of substitutions in the prenyl pocket on the energy of formation of the Pdc·G$\beta\gamma$ complex in the presence or absence of the prenyl group. We find that the prenyl group does contribute to the Pdc·G$\beta\gamma$ interaction as do residues involved in the conformational change and formation of the prenyl pocket. The PhLP·G$\beta\gamma$ interaction is also sensitive to these changes in G$\beta\gamma$ but to a lesser degree, suggesting a smaller contribution of the prenyl group to the binding of PhLP.

EXPERIMENTAL PROCEDURES

Construction of Vectors, Protein Expression, and Purification. Rat Pdc-myc-His_6 and PhLP-myc-His_6 constructs were inserted into the bacterial expression vector pET15b (Novagen) as described previously (*41*, *48*). Both constructs were expressed in *Escherichia coli* DE3 cells and purified by Ni^{2+} affinity chromatography under nondenaturing conditions as described (*48*). The purified proteins were concentrated and buffer-exchanged by ultrafiltration into storage buffer (20 mM HEPES, pH 7.2, 150 mM NaCl) and stored in 50% glycerol at −20 °C. Protein concentrations were determined using the Coomassie Plus protein assay reagent (Pierce), and the purity of the proteins was determined by SDS−PAGE to be 90−95%.

The procedures for construction of recombinant baculoviruses for native G protein β and γ subunits have been described (*49*). The six "prenyl pocket" mutations in the Gβ_1 subunit were made using PCR-based site-directed mutagenesis, and the cDNAs were cloned into the baculovirus transfer vector, pVL1393, and sequenced as described (*9*, *47*, *50*, *51*). Recombinant baculoviruses were made using published procedures (*49*). Viruses encoding for native or mutated γ subunits were expressed with the Gβ_1 subunit, and the

expressed proteins were purifed by a modification of the method described by Kozasa (*52*). Briefly, Sf9 cells were infected with the His_6-$G_i\alpha_1$-containing baculovirus at a multiplicity of infection of 3 along with the desired β and γ baculoviruses and harvested 48−60 h after infection. Membranes were prepared from the Sf9 cells and extracted with 0.1% Genapol. Extracts were passed over a Ni-NTA column and washed with Ni-NTA base buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 μM GDP) containing 17 μg/mL PMSF, 2 μg/mL pepstatin, leupeptin, aprotinin, 5 mM imidazole, 0.1% Genapol, and 500 mM NaCl. A subsequent wash with Ni-NTA base buffer containing 0.1% CHAPS was performed, followed by elution of protein with Ni-NTA base buffer containing 50 mM $MgCl_2$, 10 mM NaF, 30 μM $AlCl_3$, and 1% cholate. Protein was concentrated using an Amicon Ultra 30000 concentrator and exchanged twice using buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and 0.1% CHAPS. The purity of these preparations was equal to published examples (*49*). This method ensures that the purified G$\beta\gamma$ is in its native state since the purification relies on the ability of the G$\beta\gamma$ to bind $G_i\alpha$. Verification of proper posttranslational processing of each Gγ subunit was accomplished by mass spectrometry as described (*9*). Mass spectra of the purified G$\beta\gamma$ isoforms demonstrated that the major mass in each spectrum was compatible with predicted masses for fully processed Gγ_1 or Gγ_2 containing the geranylgeranyl or farnesyl modifications with cleavage of the three C-terminal amino acids and a carboxymethylated C-terminus. The dimer containing $\beta_1\gamma_2$-C68S was a kind gift from the laboratories of Drs. T. Kendall Harden and John Sondek, University of North Carolina. It was prepared as described (*53*). The γ subunit from the purifed $\beta\gamma$ dimer was analyzed by mass spectrometry to verify the mutation, the lack of a prenyl group, and the retention of the three C-terminal amino acids.

Co-immunoprecipitation. G$\beta\gamma$ variants (100 nM) with indicated amino acid substitutions were mixed with phosducin-myc-His_6 or PhLP-myc-His_6 (100 nM) in binding buffer (20 mM HEPES, 100 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 0.2 mM PMSF, 0.1% CHAPS) and incubated 30 min at 23 °C. Anti-myc antibody (1.4 μg; Biomol) was added and incubated for 30 min at 4 °C, after which protein A/G beads (10 μL of a 50% slurry; Santa Cruz) were added and incubated with mixing for an additional 30 min at 4 °C. The beads were pelleted and washed three times with 200 μL of binding buffer. The washed pellets were solubilized in 20 μL of 4× Laemmli sample buffer, and 12 μL was subjected to SDS−PAGE and immunoblotting with an anti-Gβ_1 antibody. Gβ_1-specific antiserum was raised in rabbits by Alpha Diagnostic International using a peptide corresponding to the N-terminal 14 residues of Gβ_1 (MSELDQLRWEAEQLC) with a C-terminal cysteine for linkage purposes. The blots were probed with a 1:2000 dilution of Gβ_1 antiserum, followed by a 1:2000 dilution of a goat anti-rabbit horseradish peroxidase secondary antibody (Bio-Rad). The blots were developed colorimetrically with 4-chloronaphthol, and the intensity of the Gβ_1 bands was determined using ImageJ software. The amount of immunoprecipitate loaded on the gel was controlled so that the Gβ_1 band intensities were within the linear range of this colorimetric detection method.

Limited Trypsin Proteolysis. Wild-type G$\beta_1\gamma_2$ or prenylless G$\beta_1\gamma_2$ at 50 μg/mL were incubated with 5 μg/mL trypsin in 25 mM Tris, pH 7.65, 75 mM NaCl, and 1 mM EDTA for 30 min at 37 °C. The reactions were quenched by the addition of 4× Laemmli sample buffer and boiling for 5 min. The digestion products were analyzed by Tricine−SDS−PAGE on 16.5% gels (*54*). The gels were stained with CYPRO Ruby protein gel stain (Bio-Rad) and were detected by illumination with UV light.

Energy Calculations. All molecular modeling was performed on SGI O2 workstations using CVFF of Accelrys InsightII/Discover. The original crystal structure (PDB ID: 1A0R) was for bovine phosducin and G$\beta_1\gamma_1$ with a farnesyl group. The amino acid sequence was modified to that of rat phosducin and human G$\beta_1\gamma_2$ in order to match the experimental proteins. One isoprene group was added at the end of the farnesyl to make a geranylgeranyl group as in γ2. The binding energy for the formation of the complex of phosducin to G$\beta\gamma$ and geranylgeranyl was calculated as $\Delta E = E_{complex} - E_{Pdc} - E_{G\beta\gamma} - E_{gg}$, where $E_{complex}$, E_{Pdc}, $E_{\beta\gamma}$, and E_{gg} are the energies of the complex, free Pdc, G$\beta\gamma$ without geranylgeranyl, and the geranylgeranyl group alone, respectively. The corresponding binding energy for the formation of the complex without geranylgeranyl was calculated as $\Delta E = E_{\beta\gamma+Pdc} - E_{\beta\gamma} - E_{Pdc}$, where $E_{\beta\gamma+Pdc}$, $E_{\beta\gamma}$, and E_{Pdc} are the energies of the complex without a geranylgeranyl binding to the pocket, the G$\beta\gamma$ without geranylgeranyl binding, and the Pdc alone, respectively. Calculated $\Delta\Delta E$ values correspond to the differences between binding energies ΔE of the modified protein and those of the wild type. All of the energies for a molecule or a complex were calculated by optimizing the residues in a predefined subset in which the residues were within the 10 Å of the geranylgeranyl group. There was no cutoff distance in calculating nonbond interaction energies. The dielectric constant was set to 4 with distant dependency.

RESULTS

Pdc and PhLP Binding to G$\beta\gamma$ Prenyl Group Variants. If the prenyl group of G$\beta\gamma$ tucks into the pocket found in the T conformation, then it should stabilize the Pdc·G$\beta\gamma$ interaction. If this is the case, then changes that affect insertion of the prenyl group into the pocket should result in changes in the binding of G$\beta\gamma$ to Pdc. To begin to investigate this possibility, binding was measured between Pdc or PhLP and variants of G$\beta_1\gamma_1$ and G$\beta_1\gamma_2$ in which the prenyl groups were switched from farnesyl to geranylgeranyl and vice versa. Prenyl group switching has been accomplished previously by changing the C-terminal residue in the CaaX motif of Gγ to leucine for geranylation and to serine for farnesylation (*9*). Binding of Pdc and PhLP to these prenyl group variants was measured by co-immunoprecipitation. Purified Pdc or PhLP with a C-terminal myc tag was added to G$\beta\gamma$, and the resulting complex was immunoprecipitated using an antibody to the myc tag. The relative amount of G$\beta\gamma$ co-immunoprecipitating with Pdc was determined by immunoblotting with an anti-Gβ_1 antibody and then quantifying the intensity of the bands on the blot. The concentrations of Pdc, PhLP, and G$\beta\gamma$ were set at 100 nM, near the reported K_d for PhLP and Pdc binding to G$\beta_1\gamma_1$ (*48*). Binding differences between wild-type and variant G$\beta\gamma$s would be roughly proportional to changes in the

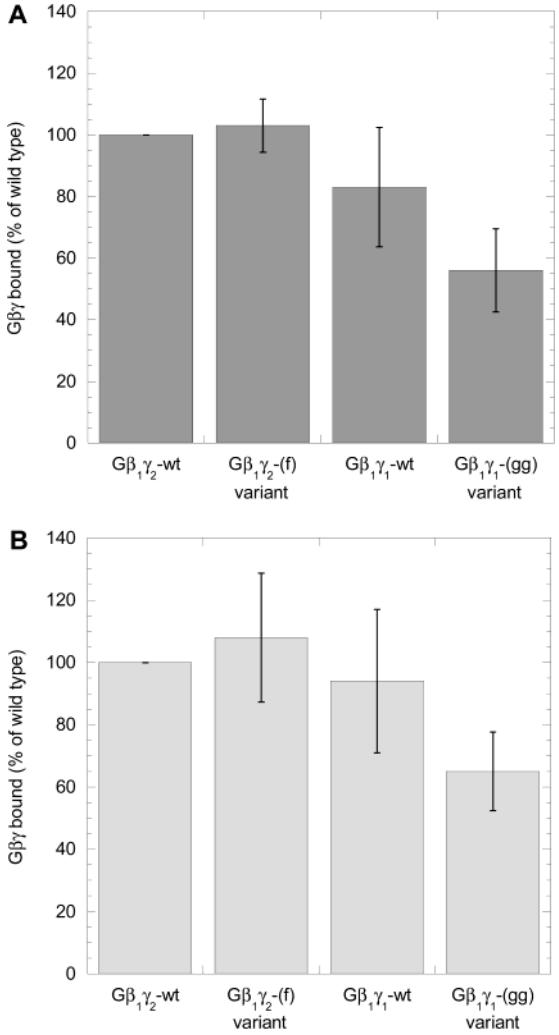

FIGURE 1: Binding of G$\beta\gamma$ prenyl group variants to Pdc and PhLP. The binding of G$\beta\gamma$ variants (100 nM) with the prenyl group changes indicated [farnesyl (f), geranylgeranyl (gg)] to Pdc (A) or PhLP (B) (100 nM) was measured by co-immunoprecipitation as described in Experimental Procedures. Binding data from six (A) or seven (B) independent experiments were combined by normalizing the binding to that of wild-type G$\beta_1\gamma_2$ and expressing it as a percent of wild type. Under these conditions, Pdc bound 46 ± 16 nM ($n = 6$) and PhLP bound 48 ± 11 nM ($n = 7$) of the 100 nM wild-type G$\beta_1\gamma_2$ added. The bars represent the mean ± standard deviation from these experiments.

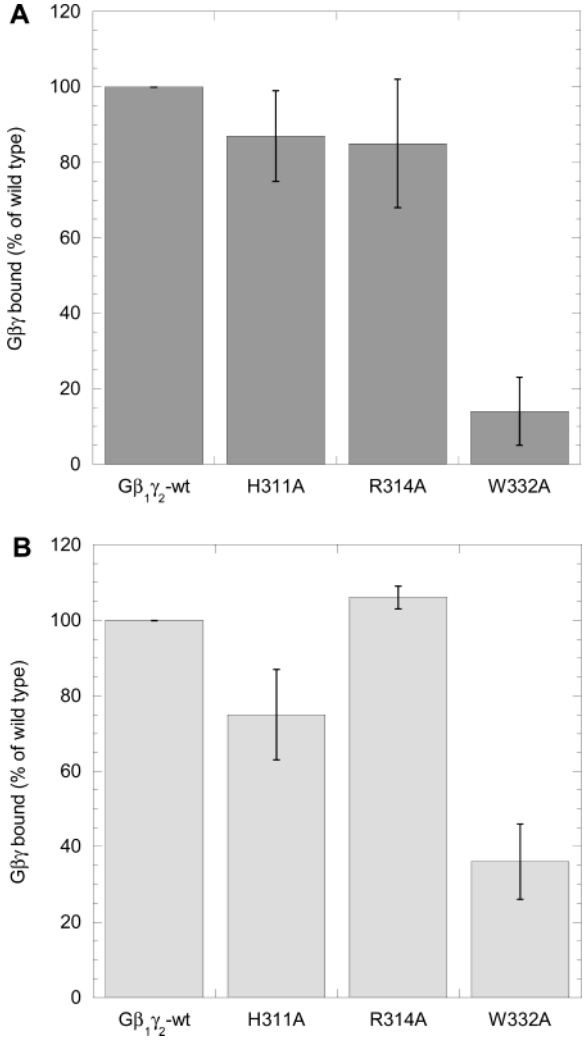

FIGURE 2: Binding of G$\beta\gamma$ conformational change variants to Pdc and PhLP. G$\beta\gamma$ variants with alanine mutations at residues that undergo significant conformational changes upon Pdc binding were tested for Pdc (A) and PhLP (B) binding as described in Figure 1. Data represent the mean ± standard deviation from three independent experiments.

binding affinity near the K_d, allowing reasonable estimates of relative differences in binding affinity to be determined with limiting amounts of the G$\beta\gamma$ variants. A full titration curve for PhLP and wild-type G$\beta_1\gamma_2$ was done using the co-immunoprecipitation method described here and also yielded an EC_{50} of 100 nM (data not shown), confirming that this method produced binding affinities similar to those determined by other methods (*48*).

Farnesylated G$\beta_1\gamma_2$ bound Pdc and PhLP as well as wild-type geranylgeranylated G$\beta_1\gamma_2$, while geranylgeranylated G$\beta_1\gamma_1$ bound Pdc and PhLP 30−35% less than wild-type farnesylated G$\beta_1\gamma_1$ (Figure 1). From these results, it appears that the type of prenyl group on Gγ_2 has no effect on G$\beta\gamma$ binding to Pdc or PhLP, while the type of prenyl group on Gγ1 has only moderate effects on binding. These findings are consistent with previous data showing only small differences in the binding of Pdc to various G$\beta\gamma$ subunit combinations in which the Gγ subunit was either farnesylated (Gγ_1) or geranylgeranylated (Gγ_2 and Gγ_3) (*55*). The results are also consistent with similar binding of G$\beta\gamma$ to $G_t\alpha$ whether the Gγ was farnesylated or geranylgeranylated (*19*). Two possible conclusions from these data are that either the two prenyl groups insert approximately as well into the pocket or that neither insert and the pocket is not occupied.

Pdc and PhLP Binding to G$\beta\gamma$ Conformational Change Variants. Three residues (H311, R314, and W332) of the Gβ subunit undergo considerable displacements between the R and T state in order to make contacts with Pdc (*43*, *45*). Single alanine substitution of each of these Gβ residues causes a 20-fold decrease in the ability of G$\beta\gamma$ to activate PLCβ or AC-II (*47*). Therefore, the binding of these conformational change variants to Pdc and PhLP was measured to assess the contribution of the conformational change and formation of the prenyl pocket in the interaction with Pdc. The H311A and R314A variants of G$\beta_1\gamma_2$ bound Pdc and PhLP equally well as wild type (Figure 2). In contrast, the W332A variant was decreased by more than 80% when binding Pdc and by more than 60% when binding PhLP. In the Pdc·G$\beta\gamma$ structure, W332 participates in extensive hydrophobic contacts with M98, M101, and L105

of Pdc, while R314 makes an electrostatic interaction with E229 of Pdc and H311 forms electrostatic interactions with E196 and K193 of Pdc (*43*, *45*). The hydrophobic contacts of W332 appear to make an important contribution to the binding of G$\beta\gamma$ to Pdc, and they may be a driving force for the conformational change in G$\beta\gamma$. On the other hand, the electrostatic contacts of H311 and R314 appear to make only minor contributions. These results do not directly address the question of binding of the prenyl group into the pocket, but they are consistent with two previous observations with respect to Pdc-induced displacement of G$\beta_1\gamma_1$ from the membrane. First, the N-terminal domain of Pdc was effective in blocking binding of G$\beta_1\gamma_1$ to the membrane in the absence of the C-terminal domain, despite the fact that there is no steric hindrance of the membrane binding surface of G$\beta_1\gamma_1$ by the N-terminal domain (*48*). It is possible that the interaction of M98, M101, and L105 and other residues within the N-terminal domain of Pdc with G$\beta\gamma$ induces the conformational change, resulting in insertion of the prenyl group into the pocket and dissociation of G$\beta\gamma$ from the membrane. Second, phosphorylation of Pdc at S106, a residue next to the G$\beta_1\gamma_1$ W332 contact site, caused a significant decrease in the ability of Pdc to block G$\beta_1\gamma_1$ binding to the membrane (*32*). Thus, there is a correlation between the R to T conformational change and the ability of Pdc to bind G$\beta\gamma$ and block its association with the membrane, possibly by burying the isoprenoid in the prenyl pocket.

Pdc and PhLP Binding to G$\beta\gamma$ Prenyl Pocket Variants. In an effort to better resolve the issue of prenyl group insertion into the pocket, binding was measured between Pdc or PhLP and variants of G$\beta\gamma$ in which amino acids that line the prenyl pocket were substituted. Five alanine substitutions (V315A, S331A, F335A, K337A, and W339A) and one nonconservative lysine substitution (T329K) were made and tested (*47*). The five alanine mutations were designed to widen the prenyl pocket while the lysine mutation was designed to shorten the pocket and add a positive charge. None of the substituted residues comes into direct contact with Pdc, so any change in binding of these variants to Pdc or PhLP must result not from a loss of binding contacts but from disruption of the T conformation. Thus, these prenyl pocket variants would be useful in determining if the prenyl group actually does insert into this groove and stabilize the T conformation of G$\beta\gamma$. Substitution of residues at the distal end of the pocket (T329K and V315A) had a significant impact on Pdc binding. The nonconservative T329K substitution decreased Pdc binding by greater than 90% (Figure 3A), while the conservative V315A substitution decreased binding by about 70%. These effects are similar to the decrease in binding caused by the W332A substitution in which several hydrophobic contacts between G$\beta\gamma$ and Pdc were lost, indicating a significant disruption of the Pdc·G$\beta\gamma$ interaction when the prenyl pocket is disturbed. The other substitutions in the pocket had moderate effects with K337A and W339A decreasing binding by 40% and S331A by 20%, while F335A had no effect. The impact of these substitutions on PhLP binding was less in all cases except for W339A, where a similar 40% decrease was observed (Figure 3B).

The cause of the decrease in binding was further investigated for the T329K variant by modeling the effect of the T329K substitution on the structure of the prenyl pocket.

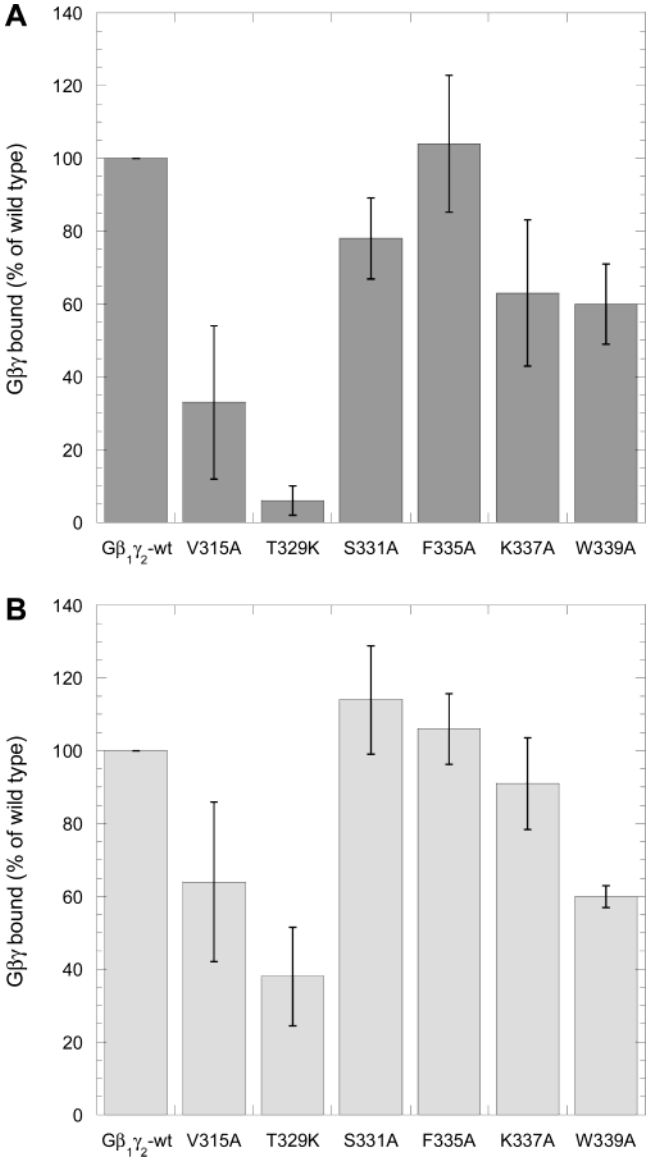

FIGURE 3: Binding of G$\beta\gamma$ prenyl pocket variants to Pdc and PhLP. G$\beta\gamma$ variants with the indicated amino acid substitutions at residues proposed to contact the prenyl group upon Pdc binding were tested for Pdc (A) and PhLP (B) binding as described in Figure 1. Data represent the mean ± standard deviation from three independent experiments.

The total conformational energy of both the T329K and the wild-type structures was first minimized in the absence of the prenyl group, and then the isoprenoid was reinserted into the pocket. The resulting structures are visualized in Figure 4. The minimization caused negligible changes in the position of the atoms around the prenyl pocket. Furthermore, the only significant difference between the structures of the wild type and the T329K variant was the occupation of the pocket by the lysine side chain. In the wild type, the T329 (shown in yellow) does not interfere with the insertion of the farnesyl group (shown in green) into the pocket (Figure 4A). However, when the lysine side chain is placed at position 329 (shown in yellow), the farnesyl group cannot fit into the pocket without considerable steric overlap from the K329 side chain (Figure 4B). This structural model suggests that the decrease in binding of Pdc and PhLP to the T329K variant was a result of an inability of the prenyl group to fit into the prenyl pocket and stabilize the T conformation and

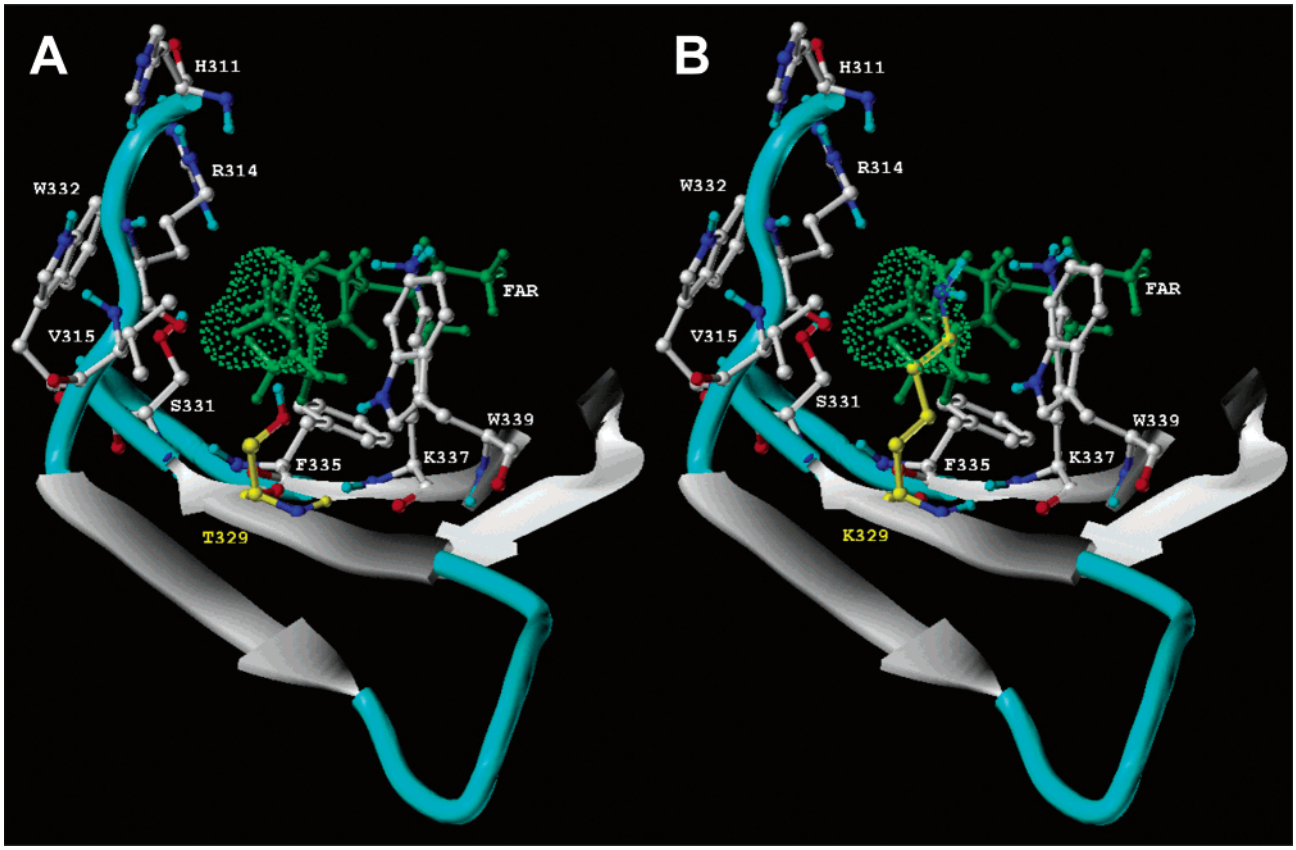

FIGURE 4: Structural comparison of the wild-type and the T329K prenyl pockets. The prenyl pockets of the wild-type Pdc·G$\beta_1\gamma_1$ complex (A) and the T329K variant (B) are shown. Side chains of residues that were substituted in this study are shown as ball-and-stick models. The carbon atoms of T329 (A) or the substituted K329 (B) are shown in yellow. The farnesyl group is shown in green. The structures were obtained starting with the crystal structure of Pdc·G$\beta_1\gamma_1$ (*38*) and either retaining the T329 side chain or replacing it with K. The farnesyl group was then extracted from the structures, and all residues located 7 Å or less around the prenyl group location were energy minimized, while the rest of the complex was fixed in an aggregate. Following the minimization the farnesyl group was merged back into the pocket. The dotted surface at the end of the farnesyl group emphasizes the steric overlap between the prenyl group and K329 in panel B. All manipulations of the structure were done using the Sybyl 6.9 software from Tripos, Inc.

was not the result of disruption in the folding of the β-propeller of G$\beta\gamma$ in this region of the structure.

Energy Calculations of Pdc Binding to G$\beta\gamma$ Prenyl Pocket Variants. To further investigate whether the prenyl pocket variants exerted their effects on Pdc·G$\beta\gamma$ binding through changes in prenyl group insertion, the binding energy of the interaction of prenyl pocket variants with Pdc in the presence or absence of the prenyl group was determined by molecular modeling calculations. The energies of dissociated Pdc, G$\beta_1\gamma_2$, and geranylgeranyl structures were calculated and subtracted from the energy of the Pdc·G$\beta_1\gamma_2$·geranylgeranyl complex to obtain a ΔE for complex formation. The resulting ΔE for the wild-type G$\beta_1\gamma_2$ was then subtracted from the ΔE of the prenyl pocket variants to give a $\Delta\Delta E$ for each variant (Figure 5A). Positive $\Delta\Delta E$ values represent complexes that are less stable than the wild type. Similar calculations were made in the absence of the geranylgeranyl group (Figure 5B). In the presence of the prenyl group, the $\Delta\Delta E$ values correlated closely with the binding measurements, with T329K having the highest $\Delta\Delta E$ value followed by V315A. In contrast, the $\Delta\Delta E$ values calculated in the absence of the prenyl group correlated poorly with the binding measurements. In particular, a negative $\Delta\Delta E$ value was calculated for T329K, suggesting that this variant would be more stable that the wild type in a Pdc·G$\beta_1\gamma_2$ complex without the geranylgeranyl moiety. The need to include the geranylgeranyl group to effectively correlate the calculated and experimental binding affinities suggests that the amino acid substitutions in the prenyl pocket exert their effect on the stability of Pdc·G$\beta_1\gamma_2$ complex through the geranylgeranyl group, supporting the idea that the geranylgeranyl group inserts into the prenyl pocket and interacts with residues in the pocket.

Pdc and PhLP Binding to Prenylless G$\beta\gamma$. To verify the contribution of the prenyl group suggested by the effects of the prenyl pocket variants, a G$\beta_1\gamma_2$ variant (G$\beta_1\gamma_2$-C68S) without a geranylgeranyl group was prepared by substituting the cysteine residue in the CaaX prenylation motif of Gγ_2 with serine, and the binding of this prenylless G$\beta\gamma$ to Pdc and PhLP was measured. Binding to both Pdc and PhLP was inhibited by greater than 90% compared to wild-type G$\beta_1\gamma_2$ (Figure 6A,B). The striking decrease in binding of G$\beta_1\gamma_2$-C68S did not appear to be a result of misfolding of the protein caused by a lack of the prenyl group, because limited trypsin proteolysis of the G$\beta_1\gamma_2$-C68S gave a degradation pattern very similar to that of wild-type G$\beta_1\gamma_2$ (Figure 6C), yielding 24 and 13 kDa fragments as reported previously for native G$\beta\gamma$ (*16*). In addition, analysis of the γ subunit from the G$\beta_1\gamma_2$-C68S dimer by mass spectrometry indicated a correct molecular mass for the mutated, nonprenylated γ subunit with retention of residues 69−71 at the C-terminus (data not shown). In the wild-type Gγ_2, these three C-terminal residues are proteolytically cleaved after prenylation followed by carboxymethylation of the C-terminus. Thus, it is possible

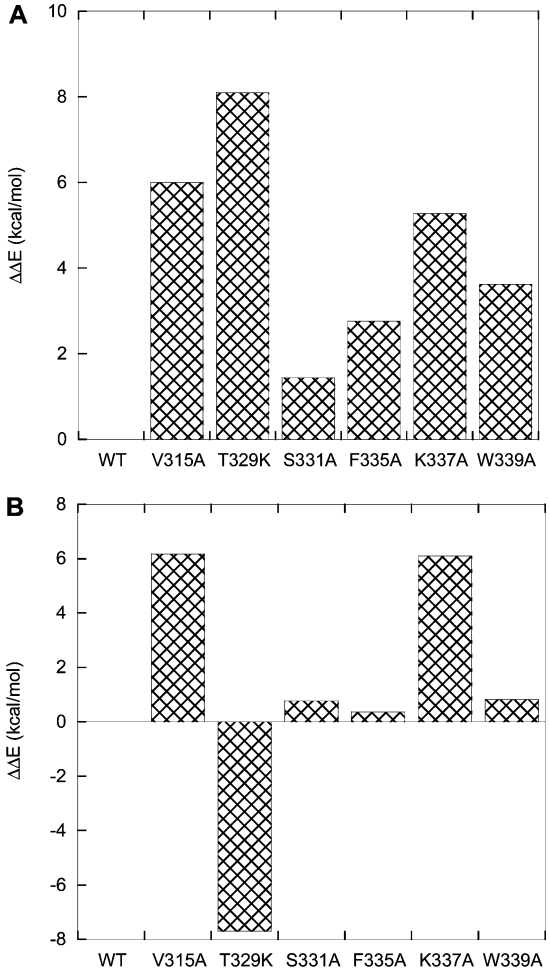

FIGURE 5: Energy calculations of the binding of Pdc to G$\beta\gamma$ prenyl pocket variants. (A) ΔE values for the formation of the Pdc·G$\beta_1\gamma_2$· geranylgeranyl complex from free Pdc, G$\beta_1\gamma_2$, and geranylgeranyl were calculated by subtracting the minimized energies of the free species from the minimized energy of the complex. $\Delta\Delta E$ for each variant was calculated by subtracting ΔE for the wild type from ΔE for each variant as indicated. (B) ΔE values for the formation of the Pdc·G$\beta_1\gamma_2$ complex without the geranylgeranyl group were calculated by subtracting the minimized energies of the free Pdc and G$\beta_1\gamma_2$ from that of the complex. $\Delta\Delta E$ values for each variant were determined as in panel A.

that these additional residues at the C-terminus also contribute to the very weak binding of G$\beta_1\gamma_2$-C68S to Pdc and PhLP. In support of this possibility, the original crystallographic structure of the Pdc·G$\beta_1\gamma_1$ complex had the prenylated cysteine and two additional C-terminal amino acids of Gγ_1 removed by digestion with endo-LysC for crystallization purposes, yet the binding affinity was sufficient to form the complex at the high protein concentration required for crystallization (*43*). In contrast, retention of these C-terminal residues had little effect on G$\beta\gamma$ activation of PLCβ (*16*, *56*). Thus, it is probable that both the lack of a prenyl group and the retention of the three C-terminal amino acids contribute to the poor binding of G$\beta_1\gamma_2$-C68S to Pdc and PhLP.

DISCUSSION

The data presented support the hypothesis that the prenyl group of Gγ inserts into the prenyl pocket on the Gβ subunit and stabilizes the T state of G$\beta\gamma$ induced by Pdc binding (*45*). Several different pieces of evidence bolster this conclusion. First, amino acid substitutions that disrupt putative contacts of the prenyl group within the pocket or that sterically block the pocket inhibit G$\beta_1\gamma_2$ binding to Pdc and PhLP. These residues do not come into direct contact with Pdc; therefore, they must destabilize the structure allosterically. The best explanation for allosteric destabilization by prenyl pocket variants is the disruption of the binding of the geranylgeranyl moiety of G$\beta_1\gamma_2$ in the pocket. Second, calculation of the energy of formation of the Pdc·G$\beta_1\gamma_2$ complex with the prenyl pocket variants requires insertion of the geranylgeranyl group into the pocket in order to fit the experimentally measured effects of the amino acid substitutions on Pdc binding. Third, a prenylless G$\beta_1\gamma_2$-C68S variant binds Pdc less than geranylgeranylated G$\beta_1\gamma_2$, suggesting that the prenyl group inserts into the pocket created by Pdc binding and stabilizes the Pdc·G$\beta\gamma$ complex. Occupation of the pocket by either a farnesyl or a geranylgeranyl isoprenoid appears to be sufficient to stabilize the T conformation (see Figure 1).

Burying of the prenyl group within the G$\beta\gamma$ structure is consistent with the fact that the Pdc·G$\beta_1\gamma_1$ complex is found in the cytosol (*27*−*29*). When G$\beta\gamma$ associates with cell membranes, the prenyl group is believed to insert into the lipid bilayer and contribute to the binding of G$\beta\gamma$ to the bilayer (*11*−*13*). Upon Pdc binding, G$\beta\gamma$ is released from the bilayer, and the C-terminal domain of Pdc covers the membrane-binding face of G$\beta\gamma$, effectively blocking reassociation of the complex with the bilayer (*43*). The hydrophobic isoprenoid would be unstable in an aqueous environment; thus its insertion into the hydrophobic pocket of G$\beta\gamma$ created by the Pdc-induced conformational change would be energetically favorable. This complex would effectively sequester G$\beta\gamma$ from the membrane where Gα and effectors such as PLCβ, AC-II, and K^+ channels are found. This ability of Pdc to dissociate prenylated G$\beta\gamma$ from the membrane is reminiscent of the ability of the Rho GTPase GDP dissociation inhibitor (Rho·GDI) to dissociate the prenylated Rho·GDP from the plasma membrane. In this case, the geranylgeranyl group of Rho is believed to insert into a hydrophobic pocket on Rho·GDI (*57*−*59*), while Pdc appears to create a hydrophobic pocket for the prenyl group on G$\beta\gamma$ by inducing the conformational change to the T state.

It has been suggested that the T conformation of G$\beta\gamma$ is the effector binding conformation (*14*, *16*, *47*). This idea is supported by the fact that amino acid substitutions of the prenyl pocket inhibit AC-II activation by G$\beta\gamma$ (*47*), while other substitutions on this same face of G$\beta\gamma$ have no effect (*46*). The data reported here do not directly address this question, but they do indicate that the prenyl moiety is tucked into the prenyl pocket of Gβ in the T conformation and is not inserted into the lipid bilayer. Most G$\beta\gamma$ effectors are membrane associated; thus it would not appear necessary to remove the prenyl group from the bilayer in order for G$\beta\gamma$ to interact with these effectors. However, if appropriate contacts between G$\beta\gamma$ and its effectors require reorientation of G$\beta\gamma$ with respect to the bilayer, then formation of the T conformation and insertion of the prenyl group into the pocket might be favorable.

In almost every case, PhLP was less sensitive than Pdc to amino acid substitutions that perturbed the conformational change or the prenyl pocket. The N-terminal domain of PhLP binds G$\beta_1\gamma_1$ with a 10-fold higher affinity than the N-

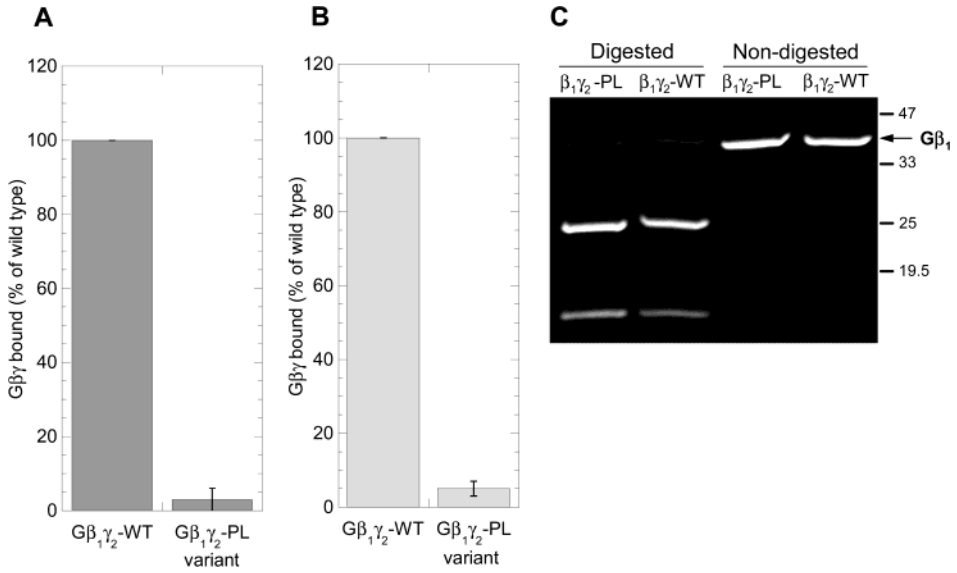

FIGURE 6: Binding of prenylless G$\beta_1\gamma_2$-C68S to Pdc and PhLP. The binding of prenylless (PL) G$\beta_1\gamma_2$-C68S to Pdc (A) or PhLP (B) was measured as described in Figure 1. Data represent the mean ± standard deviation from three independent experiments. (C) The native state of the prenylless G$\beta_1\gamma_2$-C68S was tested by limited trypsin digestion. G$\beta_1\gamma_2$-C68S or wild-type G$\beta_1\gamma_2$ was digested with trypsin and analyzed by SDS−PAGE as described in Experimental Procedures. A CYPRO-Ruby stained gel of both the nondigested (0.5 μg per lane) and the digested proteins (0.85 μg per lane) is shown.

terminal domain of Pdc, with a K_d of 0.5 μM compared to a K_d of 5 μM for Pdc, while full-length PhLP and Pdc have similar K_d values, 0.1 and 0.05 μM, respectively (*48*). Thus, it appears that PhLP derives more binding energy from interactions within the N-terminal domain that are not involved in the conformational change while Pdc relies more on contacts created by the conformational change. Interestingly, the residues of Pdc that bind G$\beta_1\gamma_1$ are highly conserved in PhLP, so it is likely that these same residues are involved in PhLP·G$\beta_1\gamma_1$ binding. Hence, different binding contacts are probably not the source of the increased contribution of the N-terminal domain of PhLP to G$\beta\gamma$ binding. A more likely source of this difference is an increased stability of the N-terminal domain of PhLP. The X-ray structural data suggest that the N-terminal domain of Pdc would be structurally unstable in the absence of G$\beta\gamma$ (*43*, *60*). PhLP, on the other hand, has a 37-residue N-terminal extension that may enhance the stability of the N-terminal domain prior to G$\beta\gamma$ binding, thereby increasing the binding affinity of the N-terminal domain by decreasing the loss of entropy that occurs when the complex forms (*48*). This increase in N-terminal stability may make PhLP binding less dependent on the G$\beta\gamma$ conformational change.

In summary, the data reported here support the proposed structural model in which binding of Pdc to G$\beta\gamma$ causes a conformational change that creates a pocket between β-propeller blades 6 and 7 of G$\beta\gamma$ into which the prenyl group inserts (*45*). As a result, the affinity of G$\beta\gamma$ for the plasma membrane decreases, allowing the C-terminal domain of Pdc to interact with the membrane-binding face of G$\beta\gamma$ and sterically block association of G$\beta\gamma$ with the membrane. In this manner, Pdc displaces G$\beta\gamma$ from the membrane into the cytosol away from its effectors, Gα, and receptors. Whether sequestration of G$\beta\gamma$ in the cytosol is a mechanism of downregulation of G protein signaling or whether further signals are propagated through the Pdc·G$\beta\gamma$ interaction remains to be seen.

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