Identification of a C-terminal binding site for G-protein βγ-subunits in phosducin-like protein

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Abstract Phosducin-like protein (PhLP) has recently been identified as a ubiquitous inhibitor of G-protein βγ-subunit ($G_{\beta\gamma}$)-mediated signaling, with an affinity about 5-fold lower than that of phosducin. The $G_{\beta\gamma}$ binding site of phosducin has been suggested to be contained in its N-terminus. A region corresponding to this N-terminus is lacking in PhLP, suggesting that PhLP must utilize a different mode of $G_{\beta\gamma}$ binding. To map the $G_{\beta\gamma}$ binding site in PhLP, a series of deletion mutants were constructed, expressed in *E. coli* as glutathione *S*-transferase (GST) fusion proteins, and the purified fusion proteins were examined for their ability to attenuate G_o GTPase activity. Progressive N-terminal truncations of PhLP caused only minor reductions in potency, whereas the complementary N-terminal PhLP fragments turned out to be inactive. We further identified a short C-terminal segment comprising residues 168 to 195 that inhibited G_o GTPase activity similar in efficacy and potency to full-length PhLP. This C-terminal fragment was also capable of antagonizing a second $G_{\beta\gamma}$-mediated function, the enhancement of rhodopsin phosphorylation by the β-adrenergic receptor kinase. Taken together, these data indicate that PhLP interacts with $G_{\beta\gamma}$ via a short C-terminal binding site which is distinct from that identified previously in phosducin.

Key words: Phosducin-like protein; G-protein βγ-subunit; β-Adrenergic receptor kinase

1. Introduction

Heterotrimeric G-proteins with their α-, β- and γ-subunits relay signals from seven-transmembrane-helix receptors to effector molecules. Upon agonist binding, receptors stimulate G-proteins to exchange GDP for GTP which leads to dissociation of GTP-liganded α-subunits from tightly bound βγ-subunit complexes (for reviews, see [1–3]). For a long time, G_α was considered to be the principal mediator of G-protein function. However, over the past years it has become evident that $G_{\beta\gamma}$-subunits have multiple signaling functions of their own (reviewed in [4–6]). The growing list of $G_{\beta\gamma}$-regulated effectors includes adenylyl cyclases, phospholipases C-β and A2, several ion channels and kinases, such as phosphoinositide-3-kinase and mitogen-activated kinase. In addition, $G_{\beta\gamma}$-subunits provide a membrane anchor and an activator for the β-adrenergic receptor kinase (βARK) which enables the kinase to translocate from the cytosol to its substrate receptors in the cell membrane [7–9].

Phosducin is a $G_{\beta\gamma}$-binding protein, for which no effector function has been reported so far. It is a cytosolic phosphoprotein that is most abundantly expressed in retinal rods [10] and the pineal gland [11], but has also been detected in many other tissues [12,13]. Phosducin binds to $G_{\beta\gamma}$ dimers with high affinity and can thereby inhibit G-protein-mediated signaling [12,14,15]. Phosducin has also been demonstrated to compete with βARK for $G_{\beta\gamma}$ and thus to attenuate βARK-catalyzed receptor phosphorylation [16,17].

$G_{\beta\gamma}$-regulated proteins have recently attracted much interest with respect to identifying common $G_{\beta\gamma}$-binding motifs. In the case of βARK and the Ras guanine nucleotide releasing factor, the $G_{\beta\gamma}$-binding region has been mapped to the C-terminus of their pleckstrin homology domain including extensions beyond it [18,19]. Furthermore, several unrelated $G_{\beta\gamma}$ effectors have been found to share a common sequence, Gln/Asn-X-X-Glu/Asp-Arg/Lys, which has been proposed to be an important determinant for receiving signals from $G_{\beta\gamma}$ [20]. In phosducin, which does not contain the structural motifs mentioned, the $G_{\beta\gamma}$-binding domain has been assigned by two independent studies to the N-terminal 105 [21] or 63 amino acids [22], respectively.

Recently, the cDNAs of two splice variants of a phosducin-like protein have been cloned from rat brain which show a broad tissue distribution [23]. The splice variants differ in the length of their N-termini. The short variant, hereafter referred to as PhLP, lacks most of the N-terminal region which in phosducin has been postulated to contain the $G_{\beta\gamma}$-binding site [21,22]. However, PhLP, when expressed and purified as a recombinant protein, did show $G_{\beta\gamma}$ binding in a variety of assays [24]: it inhibited the GTPase activity of G_o as well as the $G_{\beta\gamma}$-subunit-dependent ADP-ribosylation of $G_{\alpha o}$ by pertussis toxin, and it antagonized the effects of $G_{\beta\gamma}$-subunits on the phosphorylation of rhodopsin by βARK. In all these assays, the affinity of PhLP was only 5-fold lower than that of phosducin. Therefore, PhLP appears to have its own mode of $G_{\beta\gamma}$ binding. The present study was undertaken to identify this $G_{\beta\gamma}$-binding site.

2. Materials and methods

2.1. Construction, expression and purification of GST fusion proteins

PhLP wild-type and deletion mutant cDNAs were amplified by polymerase chain reactions using the complete coding sequence of PhLP as a template [24] and 5′ and 3′ primers that contained *Bam*HI and *Eco*RI sites, respectively. The various cDNA fragments were ligated in-frame downstream from the GST coding region and the thrombin cleavage site of the fusion vector pGEX-1λT (Pharmacia). All cDNA constructs were confirmed by automated sequencing and were introduced into *E. coli* strain BL21(DE3)pLysS. Following in-

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Abbreviations: G_α, G-protein α-subunits; G_β, G-protein β-subunits; $G_{\beta\gamma}$, G-protein βγ-subunits; GST, glutathione *S*-transferase; PhLP, phosducin-like protein (short variant); βARK, β-adrenergic receptor kinase-1; Mas-7, mastoparan analogue (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Ala-Leu-Leu-NH_2)

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duction with 0.1 mM isopropyl-β-D-thiogalactoside for 2.5 h at 25°C, the cell pellets were disrupted in a buffer containing 50 mM Tris-HCl, pH 7.8, 20 mM EDTA and 0.1% Triton X-100. DNA was precipitated with 2% streptomycin sulfate followed by centrifugation for 30 min at 50 000 × *g*. The clear supernatant was applied on a glutathione agarose (Fluka) column and washed with 20 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1 mM EDTA and 1 mM DTT. The GST fusion proteins were eluted with washing buffer containing 10 mM glutathione (Fluka), pH 7.6, and were dialyzed against 10 mM Tris-HCl, pH 7.6, 1 mM DTT at 4°C. The fractions were concentrated in Centricon devices (Amicon), glycerol was added to a final concentration of 15%, and samples were stored at −80°C.

2.2. Purification of thrombin-cleaved fusion proteins

GST fusion protein bound to glutathione agarose beads (Fluka) was cleaved with 20 units of thrombin (Sigma) per 5 mg fusion protein in a buffer containing 20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2.5 mM $CaCl_2$, 1 mM DTT. Following 3 h of incubation at room temperature, cleaved PhLP and thrombin were eluted. Contaminating thrombin was removed by binding to SP-Sepharose (Pharmacia). Fractions of unbound PhLP were desalted, concentrated and stored as described in Section 2.1.

2.3. Purification of other proteins

G_o and G-protein βγ-subunits were purified from bovine brain according to established procedures [25]. Bovine βARK was expressed in Sf9 cells and purified as described [26].

2.4. Protein determination

The purity of proteins was judged from Coomassie-blue-stained polyacrylamide gels and their concentration determined according to Bradford [27].

2.5. GTPase activity of G_o

G_o was reconstituted into crude soybean phosphatidylcholine vesicles as described [28]. The GTPase activity of 0.1 pmol (1 nM) G_o was determined in a volume of 100 μl in the presence of 50 μM of Mas-7 (Biomol) as described [12,29]. The effects of fusion proteins containing full-length and mutant PhLP were examined at concentrations of 3–3000 nM.

2.6. Phosphorylation of rhodopsin

Urea-treated rod outer segments containing >95% rhodopsin were prepared from bovine retina [30]. 40 pmol rhodopsin was phosphorylated in a volume of 40 μl using 7.5 nM purified recombinant βARK and 75 nM purified G-protein βγ-subunits as described earlier [16]. The C-terminal PhLP fragments were present at 1, 5 and 20 μM. The incubation was carried out at 30°C for 6 min under bright white light. The reaction was stopped, and the samples were analyzed by SDS polyacrylamide gel electrophoresis, autoradiography, and Cerenkov counting of the excised rhodopsin bands.

2.7. Data analysis

Data are presented as means ± S.E.M. of at least three independent experiments. Concentration-response curves were fitted to the Hill equation by non-linear curve-fitting as described earlier [31] in order to obtain estimates for potency (IC_{50}) and efficacy (I_{max}) of PhLP and its deletion constructs.

3. Results

In order to map rat PhLP for regions that are involved in binding to $G_{\beta\gamma}$ we prepared a series of deletion mutants using PCR (Fig. 1). Wild-type PhLP and its mutants were expressed as GST fusion proteins in *E. coli*. Following cell disruption and centrifugation, fusion proteins were obtained in the supernatant. Best yields of soluble protein were achieved when bacterial expression was induced at 25°C with low concentrations of isopropyl-β-D-thiogalactoside (0.1 mM) followed by lysing the cells with Triton X-100. From the soluble fraction, GST fusion proteins were then purified on a glutathione agarose column. The resultant preparations were >95% pure as judged by Coomassie-blue-stained SDS-polyacrylamide gels (not shown).

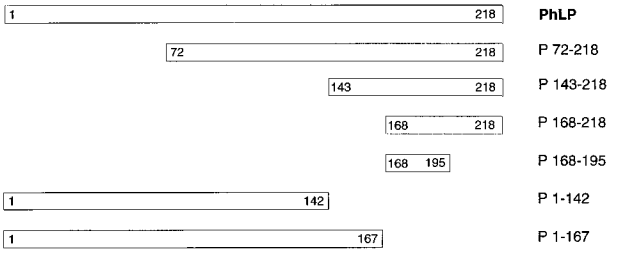

Fig. 1. PhLP deletion mutants. Schematic representation of full-length PhLP and six truncation mutants which were fused to glutathione *S*-transferase and expressed in *E. coli* as described in Section 2. The scheme depicts the PhLP moieties of the corresponding fusion proteins. N-terminal GST affinity tails are not shown. Numbers indicate the first and last amino acid residues in each construct according to residue positions in wild-type PhLP.

To examine the ability of the purified PhLP mutants to interact with $G_{\beta\gamma}$, we first assayed their inhibitory effect on G_o function in GTPase assays. In the presence of G-protein activators, such as receptors or high concentrations of Mg^{2+}, $G_{\beta\gamma}$ subunits enhance the GTPase activity of $G_{\alpha o}$. We reconstituted G_o purified from bovine brain into phospholipid vesicles and stimulated its GTPase activity with Mas-7, a potent analogue of mastoparan which acts on G-proteins in a receptor-mimetic way [32]. In these experiments, 50 μM Mas-7 enhanced G_o GTPase activity about 14-fold. Addition of the GST fusion protein containing the entire PhLP inhibited the activity by ~90% in a concentration-dependent manner (Fig. 2). Inhibition occurred with an IC_{50} value of 65 ± 9 nM, which is in very good accordance with the value measured for PhLP lacking the GST moiety [24]. This suggests that GST when linked N-terminally to full-length PhLP does not impair PhLP's interaction with G_o. Purified GST alone, used as a negative control, displayed no inhibitory activity in this assay (data not shown). Fig. 2 summarizes the effects of PhLP mutants that were truncated at their N-terminus. Deletions of the first 71 and 142 amino acids (GST fusion proteins of P72–218 and P143–218) caused only modest reductions of IC_{50} values by factors ~2 and ~4, respectively. However, in both cases, the maximal inhibition was smaller and reached a level of only ~70%. Further N-terminal truncations did not further shift the inhibition curves to higher concentrations: GST fusion proteins containing the very C-terminus of PhLP (P168–218) or an even shorter stretch lacking the last amino acids of the C-terminus (P168–195) still inhibited G_o function potently. Maximal inhibition for the latter fusion proteins was ~60%.

For control purposes we tested two PhLP deletion mutants that were complementary to the active C-terminal segments. The data shown in Fig. 3 illustrate that the two GST fusion proteins encompassing the first 142 or 167 amino acids failed to attenuate effectively the G_o GTPase reaction. Taken together, these observations indicate that the C-terminal segment of PhLP comprising residues 168–195 plays a crucial role in binding to G_o.

In order to obtain more direct evidence that the C-terminus of PhLP binds to G-protein βγ-subunits, we used an assay that did not involve G-protein α-subunits. To this end, we measured the enhancement of βARK-catalyzed rhodopsin phosphorylation by $G_{\beta\gamma}$-subunits. In this assay, the $G_{\beta\gamma}$-sub-

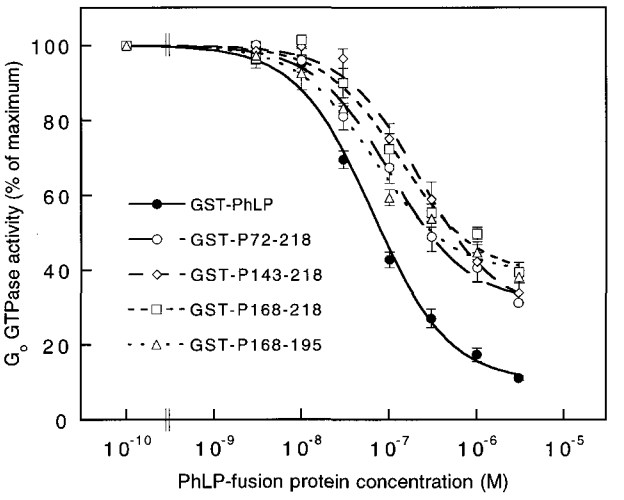

Fig. 2. Inhibition of G_o GTPase activity by GST fusion proteins containing full-length and N-terminally truncated PhLP. The GTPase activity of 0.1 pmol G_o (1 nM) reconstituted in phosphatidylcholine vesicles was determined in the presence of 50 μM Mas-7. GST-PhLP fusion proteins were present at the indicated concentrations. Values are expressed as % of the GTPase activity in the presence of Mas-7. Non-linear curve fitting to the Hill equation as described [31] gave the following IC_{50} values: GST-PhLP, 65±9 nM; GST-P72–218, 104±2 nM; GST-P143–218, 224±3 nM; GST-P168–218, 135±3 nM; GST-P168–195, 67±2 nM. Data are means ±S.E.M., $n=6$.

units serve as membrane anchors and presumably also as activators of βARK [7–9]. We have reported earlier that PhLP inhibits rhodopsin phosphorylation in this assay by up to 77±10% with an IC_{50} value of 600±200 nM [24]. Since the GST fusion moiety appeared to perturb these assays (data not shown), they were done with the PhLP fragments cleaved from GST with thrombin and then purified. The two C-terminal fragments P168–195 and P168–218 both inhibited rhodopsin phosphorylation in a concentration-dependent manner (Fig. 4) by up to 60% (at 20 μM). Half-maximal inhibition occurred at ~5 μM. The efficacy of the two peptides was thus very similar to that of the entire PhLP. This confirms the notion that the major site of interaction with G-protein βγ-subunits is contained in the region 168–195 of PhLP.

4. Discussion

G-protein βγ-subunits have recently been identified as active signal transducers. Their interactions with multiple upstream receptors as well as downstream effectors have raised considerable interest in recent years. However, there is no consensus regarding the structural or sequence requirements for such interactions. In the case of phosducin, two independent studies have mapped the $G_{\beta\gamma}$-binding domain to the N-terminus [21,22], and the N-terminus of phosducin has been proposed to contain similarities to the pleckstrin homology domain of the β-adrenergic receptor kinase [21]. Since PhLP is similar to phosducin but lacks this N-terminus, it would be expected to be devoid of $G_{\beta\gamma}$-binding. However, we have recently observed that PhLP has inhibitory effects in several assays of $G_{\beta\gamma}$ function with an affinity only about 5-fold lower than that of phosducin [24]. From these data we concluded that the $G_{\beta\gamma}$-binding domain of PhLP is most likely contained in its central and C-terminal domains, i.e. the regions showing significant similarity to phosducin, but not in its short N-terminus, which is divergent from phosducin.

In the present study we have mapped this putative $G_{\beta\gamma}$-binding site to a C-terminal fragment of PhLP comprising only 28 amino acids. This short fragment, similar in size to a peptide contained in adenylyl cyclase 2 and proposed to represent its $G_{\beta\gamma}$-binding site [20], has a submicromolar affinity for $G_{\beta\gamma}$ and an efficacy in inhibiting the GTPase activity of G_o which almost equals that of intact PhLP. We have not found any sequence similarity between the region PhLP168–195 and this QEHA peptide from adenylyl cyclase 2 (data not shown), suggesting that the two $G_{\beta\gamma}$-binding sites are different in type and presumably also in their mode of interaction with $G_{\beta\gamma}$. However, like the QEHA peptide, the PhLP fragment not only inhibits the GTPase activity of G_o – which it presumably does by disturbing the interaction between $G_{\alpha o}$ and $G_{\beta\gamma}$ – but also antagonizes the effects of $G_{\beta\gamma}$ on βARK. This indicates that via a short defined binding site PhLP can inhibit several functions of G-protein βγ-subunits. Thus, several proteins appear to be capable of impairing $G_{\beta\gamma}$ signalling properties due to distinct types of $G_{\beta\gamma}$-binding.

Gaudet et al. have very recently published the crystal structure of the complex of phosducin with transducin $G_{\beta\gamma}$ [33]. This structure reveals two separate $G_{\beta\gamma}$-binding domains of phosducin at its N- and C-terminus, respectively, that bind to spatially distinct regions on the $G_{\beta\gamma}$ surface. Interestingly, the C-terminal domain interacts with the outer strands of the transducin G_β propeller which are not involved in G_α binding. Thus, if one assumes a similar structure for PhLP, these results suggest that the C-terminal peptides of PhLP appear to inhibit G_o GTPase activity by an allosteric mechanism rather than by competition with G_α.

Taken together, our data indicate that PhLP binds to $G_{\beta\gamma}$ via a site distinct from that identified in earlier mapping studies with phosducin. This site confers on PhLP high affinity for $G_{\beta\gamma}$ and the ability to disrupt different $G_{\beta\gamma}$ functions. This high affinity for $G_{\beta\gamma}$ together with the fact that PhLP is ex-

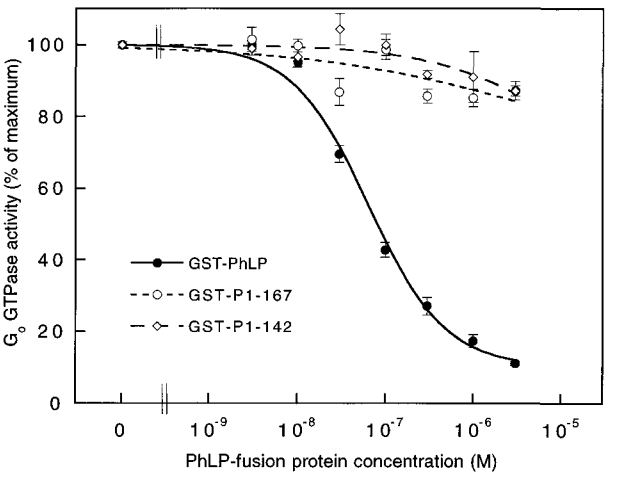

Fig. 3. Lack of effect of C-terminally truncated PhLP variants on the GTPase activity of G_o. The G_o GTPase activity was determined in the presence of 50 μM Mas-7 as in Fig. 2. GST fusion proteins comprising the N-terminal 142 and 167 amino acid residues of PhLP were assayed at increasing concentrations for their interference with G_o GTPase function. The plot shows the data obtained in comparison with the inhibition caused by the full-length PhLP fusion protein. Data are means±S.E.M., $n=6$.

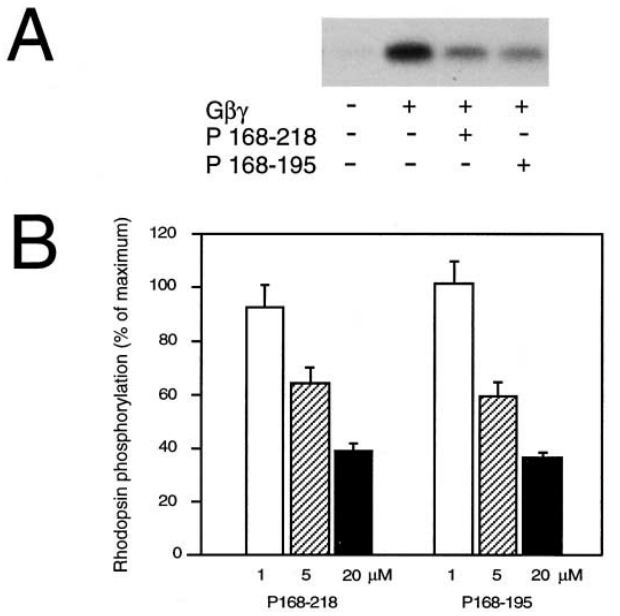

Fig. 4. Inhibition of $G_{\beta\gamma}$-enhanced rhodopsin phosphorylation by C-terminal peptides of PhLP. Rod outer segments (40 pmol rhodopsin) were phosphorylated by purified βARK (7.5 nM) in the presence of 75 nM $G_{\beta\gamma}$-subunits and varying amounts of the PhLP fragments P168–218 or P168–195, respectively, which lacked the GST moiety due to thrombin cleavage (for preparation protocol see Section 2.2). A: Autoradiogram with rhodopsin bands phosphorylated by βARK in presence of the indicated components. PhLP peptides were applied at a concentration of 20 μM. B: Quantitation of rhodopsin phosphorylation at increasing PhLP peptide concentrations. Phosphate incorporation into rhodopsin in the absence of $G_{\beta\gamma}$ (0.0071 ± 0.0006 mol phosphate per mol rhodopsin) was set at 0%, the corresponding value in the presence of $G_{\beta\gamma}$-subunits (0.051 ± 0.003 mol phosphate per mol rhodopsin) was set at 100%. Data are means ± S.E.M., $n = 6$.

pressed in many tissues suggests that it may be a major regulator of G-protein-mediated signalling by hormone and neurotransmitter receptors.

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