Interactions of Phosducin with the Subunits of G-Proteins

BINDING TO THE \( \alpha \) AS WELL AS THE \( \beta\gamma \) SUBUNITS*

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The high affinity interactions of phosducin with G-proteins involve binding of phosducin to the G-protein \( \beta\gamma \) subunits. Here we have investigated whether phosducin interacts also with G-protein \( \alpha \) subunits. Interactions of phosducin with the individual subunits of \( G_{\alpha} \) were measured by retaining phosducin-G-protein subunit complexes on columns containing immobilized anti-phosducin antibodies. Both the \( \alpha \) and the \( \beta \) subunits of trimeric \( G_{\alpha} \) were specifically retained by the antibodies in the presence of phosducin. This binding was almost completely abolished for both subunits following protein kinase A-mediated phosphorylation of phosducin and was reduced, more for \( \alpha \) than for \( \beta \) subunits, by the stable GTP analog guanosine 5'-3(3-O-thio)triphosphate. Isolated \( \alpha \) was also retained on the columns in the presence of phosducin but not in the presence of protein kinase A-phosphorylated phosducin. Likewise, purified G-protein \( \beta\gamma \) subunit complexes as well as purified \( \alpha \) subunits of \( G_{\alpha} \) and \( G_{\beta} \) were precipitated together with His6-tagged phosducin with nickel-agarose; this co-preparation occurred concentration-dependently, with apparent affinities for phosducin of 55 nm (\( G_{\beta\gamma} \)), 110 nm (\( \alpha \)), and 200 nm (\( \alpha \)). In functional experiments, the steady state GTPase activity of isolated \( \alpha \), was inhibited by phosducin by \( \approx 60\% \) with an IC\textsubscript{50} value of \( \approx 300 \) nm, whereas the GTPase activity of trimeric \( G_{\alpha} \), was inhibited by \( \approx 90\% \) with an IC\textsubscript{50} value of \( \approx 10 \) nm. Phosducin did not inhibit the GTP-hydrolytic activity of isolated \( \alpha \) as measured by single-turnover assays, but it inhibited the release of GDP from \( \alpha \); the rate constant of GDP release was decreased \( \approx 40\% \) by 500 nm phosducin, and the inhibition occurred with an IC\textsubscript{50} value for phosducin of \( \approx 100 \) nm. These data suggest that phosducin binds with high affinity to G-protein \( \beta\gamma \) subunits and with lower affinity to G-protein \( \alpha \) subunits. We propose that the \( \alpha \) subunit-mediated effects of phosducin might increase both the extent and the rapidity of its inhibitory effects compared with an action via the \( \beta\gamma \) subunit complex alone.

Heterotrimeric GTP-binding proteins (G-proteins) comprise a family of regulatory proteins that couple transmembrane receptors for a variety of neurotransmitters, hormones, and other stimuli to their intracellular effectors (for reviews see Refs. 1–4). G-proteins are composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits. Upon activation by agonist, receptors couple to their G-proteins and promote the exchange of bound GDP for GTP. The resultant conformational change induces dissociation of the GTP-bound \( \alpha \) subunit (\( G_{\alpha} \)) from the \( \beta\gamma \) subunit complex (\( G_{\beta\gamma} \)). In this dissociated state \( G_{\alpha} \) activates effectors such as adenylyl cyclases, other enzymes, or ion channels. Additionally, the free \( \beta\gamma \) subunits also interact with and regulate effectors (reviewed in Refs. 2 and 5). The intrinsic GTPase activity of \( G_{\alpha} \) then leads to hydrolysis of the bound GTP, and this enables reassociation of GDP-bound \( G_{\alpha} \) with \( G_{\beta\gamma} \). The resulting reformation of the trimeric G-protein terminates the signal.

A similar GTPase-switch function occurs in the small molecular weight monomeric GTP-binding proteins. For these proteins a large array of regulatory proteins have been identified that regulate various steps in the GTPase cycle (6). More recently, regulatory proteins have also been discovered for the heterotrimeric G-proteins. Among these are the growth cone protein GAP-43 and the RGS (regulators of G-protein signaling) family members that have been shown to activate the GTPase activity of several G-protein \( \alpha \) subunits (7–10) and a number of proteins that contain the structural motif of the pleckstrin homology domain and that bind to G-protein \( \beta\gamma \) subunits (11), most notably the \( \beta\)-adrenergic receptor kinases (12–15).

Phosducin is another type of regulator of G-protein signaling that is thought to act via the \( \beta\gamma \) subunits of G-proteins (16, 17). It was initially discovered as a major retinal phosphoprotein that could be copurified with the \( \beta \) and \( \gamma \) subunits of transducin, \( G_{\alpha} \) (18). Its expression had been thought to be restricted to the retina and the developmentally related pineal gland (18–20), but more recently phosducin has been shown to be ubiquitously expressed (16, 21).

The molecular mechanisms of the interaction of phosducin with G-proteins are not clear. The co-purification of phosducin from the retina with the \( \beta\gamma \) subunit complex of \( G_{\alpha} \) (18) indicated high affinity interactions between these proteins, and both the \( N \) and the \( C \) terminus of phosducin appear to contain high affinity binding sites for the \( \beta\gamma \) subunit complex (22–25). From these functional as well as structural observations it has been concluded that phosducin interacts exclusively with the \( \beta\gamma \) subunits of G-proteins. Indeed, studies on the effects of phosducin and phosducin-like protein on \( G_{\alpha} \) suggest that phosducin acts by “trapping” free \( \beta\gamma \) subunits; the GDP-bound \( \alpha \) subunits would then be unable to find free \( \beta\gamma \) subunits for reassociation and would therefore stay inactive (17, 26, 27).

In contrast to the many studies on phosducin-G\( G_{\beta\gamma} \) interactions, potential interactions with G-protein \( \alpha \) subunits have not been investigated. Such direct effects on \( \alpha \) subunits may be

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1 The abbreviations used are: \( G_{\alpha} \), G-protein \( \alpha \) subunit; \( G_{\beta\gamma} \), G-protein \( \beta\gamma \) subunit complex; GTP\( \gamma\S \), guanosine 5’-3(3-O-thio)triphosphate; PKA, protein kinase A; Ni-NTA, nickel-nitritolriacetic acid.
proposed from the observation that the extent of inhibition of $G_o$ GTPase is often larger than would be expected from antagonism of the function of $\beta\gamma$ subunits alone. A possible solution to this problem would be a direct interaction of phosducin with $\alpha$ subunits. Therefore, the present study was undertaken to define the nature of interactions of phosducin with the subunits of G-proteins, using mostly $G_o$ as a model system.

**EXPERIMENTAL PROCEDURES**

**Protein Purification—**Recombinant phosducin was expressed in Escherichia coli as described earlier using the plasmid pET-phosducin (16). Following lysis of the bacteria by freeze thawing and precipitation of DNA with 2% streptomycin and centrifugation at 50,000 $\times$ g for 10 min, the supernatant was applied to a MonoQ column (Amersham Pharmacia Biotech) and eluted with a 0–500 mM NaCl gradient in 10 mM Tris-HCl, pH 7.4. Peak fractions of phosducin eluting at $\approx$250 mM NaCl were concentrated to $\approx$1 ml and were then further purified by gel filtration on Superdex 200 (Amersham Pharmacia Biotech). The purity of the preparations was $>95\%$ as determined by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue. Mock preparations from E. coli transformed with "empty" vector were used as controls as described earlier (16).

Alternatively, phosducin was expressed in E. coli with a C-terminal His$_6$-tag and purified on Ni-NTA-agarose (Qiagen) as recently described (31). This preparation was used in apparently pure preparations as judged by Coomassie-stained SDS-polyacrylamide gels.

$G_o$ and its resolved $\alpha$ and $\beta\gamma$ subunits were isolated by affinity chromatography (21, 32). An antiserum against phosducin was generated by immunization of rabbits with purified recombinant phosducin (24). Columns—Phosphate/mol phosducin used in the present experiments contained 0.8–1.0 mol phosphate/mol phosducin.

**Phosphorylation of Phosducin—**The catalytic subunit of protein kinase A was purified to apparent homogeneity from bovine heart by the method of Sugden et al. (30). Phosducin in the phosducin/G-protein binding assays we used immobilization as described by Freissmuth and Gilman (29). Contaminating $\beta\gamma$ subunits were removed from the $\alpha$ preparation by a second round of heptylamine-Sepharose chromatography. The absence of contaminating $\beta\gamma$ subunits from these $\alpha$ preparations was verified in Western blots using a $\beta$monoo antibody kindly provided by Dr. G. Schultz (Department of Pharmacology, Free University, Berlin, Germany). The $\alpha$ and $\beta\gamma$ subunits of $G_o$ were purified to apparent homogeneity from bovine retina according to a protocol adapted from Gierschik et al. (33). As an alternative to antibody immobilization of phosducin, $G_o$ or $G_s$ was assayed in buffer containing 0.1% Lubrol and 25 mM MgSO$_4$. Phosducin was present at various concentrations. The incubations lasted for 30 min at 30°C and were terminated by addition of 500 $\mu$l of 1% charcoal in 2 mM Na$_2$HPO$_4$, pH 2.

The catalytic activity of $\alpha$ was measured in single-turnover assays adapted from Freissmuth and Gilman (29). $\alpha$ (1 pmol/time point) was incubated with 1 $\mu$m [gamma$^{32}$P]GTP (5 $\times$ 10$^5$ cpm/time point) in a buffer containing 50 mM Hepes, pH 7.6, 1 mM EDTA, 0.2 mM GTP, 1 mg/ml bovine serum albumin, 0.2 mM dithiothreitol, and 0.1% Lubrol in a final volume of 50 mM KCl, 6.5 mM Na$_2$HPO$_4$, pH 7.3 for 30 min at 4°C. The mixture was centrifuged at 14,000 rpm for 10 min, and 40 $\mu$l of Ni-NTA resin (Qiagen) were added to the supernatant to bind phosducin-His$_6$ (plus associated proteins). After 10 min at 4°C, the resin was pelletted by centrifugation and washed twice in the same buffer with intervening short centrifugations. G-protein subunits retained together with phosducin-His$_6$ were detected by taking up the beads in SDS-sample buffer followed by SDS-polyacrylamide gel electrophoresis and Western blotting as above.

**Data Analysis—**Kinetic data were fitted assuming exponential functions as described earlier (34). Concentration response curves were fitted to the Hill equation, and binding data were analyzed with a nonlinear binding analysis program as described (34, 35).

The concentration response curve of the GTPase inhibition of $G_o$ by phosducin (see Fig. 6) was also analyzed assuming an interaction of phosducin with both the $\alpha$ subunit and the $\beta\gamma$ subunit complex.

$$A = 100 - \{I_{95\%}P(K_o + P) + I_5\%P(K_o + P)\}$$

(Eq. 1)

where $A$ denotes the GTPase activity (in the percentage of the control activity), $I_{95\%}$ and $I_5\%$ denote the maximal inhibition caused via the $\alpha$ or $\beta$ subunits, respectively, $K_o$ and $K_d$ denote the affinities of phosducin for the $\alpha$ or $\beta\gamma$ subunits, respectively, and $P$ is the concentration of phosducin. All data are derived from at least three independent experiments and represent means $\pm$ S.E. unless stated otherwise.
To demonstrate directly the binding of phosducin to the subunits of $G_\alpha$, we incubated phosducin with $G_\alpha$ or $\alpha_\gamma$ in solution and then immobilized phosducin to detect which subunits were bound to phosducin. This was done by covalently coupling phosducin-specific antibodies raised in rabbits to protein A-Sepharose, which allowed specific retention of phosducin (and proteins bound to phosducin) on small columns. In preliminary experiments we verified that the antibodies showed no cross-reactivity with any of the subunits of $G_\alpha$, so that the $G_\alpha$ subunits should only be retained on the columns if they were bound to phosducin. Phosducin (plus associated proteins) was then eluted from the columns with 100 mM glycine-HCl, pH 2.5, and the eluates were analyzed for the presence of $\alpha$ and $\beta$ subunits of $G_\alpha$ by Western blotting. The data shown are representative of three to five independent experiments. A, specificity of the binding reaction. $G_\alpha$ (200 pmol) was incubated with (+) or without (−) an equal amount of phosducin and then loaded onto the anti-phosducin antibody column. The left lanes contain a mock preparation from E. coli with empty vector (−) indicate the nonspecific retention of $\alpha$ and $\beta$ subunits to the column, and the right lanes indicate the phosducin-dependent retention. B, inhibition of $G_\alpha$-phosducin binding by PKA-mediated phosphorylation of phosducin. Phosducin was phosphorylated by PKA (+) or incubated under identical conditions without PKA (−) prior to addition of $G_\alpha$. Incubation and retention were done as in A. C, effects of the dissociation of $G_\alpha$ induced by GTP$\gamma$S on the binding to phosducin. $G_\alpha$ was preincubated with 100 $\mu$M GTP$\gamma$S for 5 min before addition of phosducin.

**RESULTS**

To demonstrate directly the binding of phosducin to the subunits of $G_\alpha$, we incubated phosducin with $G_\alpha$, or $\alpha_\gamma$, in solution and then immobilized phosducin to detect which subunits were bound to phosducin. This was done by covalently coupling phosducin-specific antibodies raised in rabbits to protein A-Sepharose, which allowed specific retention of phosducin (and proteins bound to phosducin) on small columns. In preliminary experiments, we verified that the antibodies showed no cross-reactivity with any of the subunits of $G_\alpha$, so that the $G_\alpha$ subunits should only be retained on the columns if they were bound to phosducin. Phosducin (plus associated proteins) was then eluted from the columns with 100 mM glycine-HCl, pH 2.5, and the eluates were analyzed for the presence of $\alpha$ and $\beta$ subunits of $G_\alpha$ in Western blots. (We did not analyze the presence of $\gamma$ subunits because a lack of high affinity antibodies and also because the $\beta\gamma$ subunit complex does not appear to dissociate under physiological conditions.)

**Fig. 1. Binding of phosducin to the subunits of $G_\alpha$.** Phosducin and $G_\alpha$ or $\alpha_\gamma$ were preincubated and then loaded onto an anti-phosducin antibody column. After washing, specifically bound proteins were eluted with 100 mM glycine-HCl, pH 2.5, and the eluates were analyzed for the presence of $\alpha$ and $\beta$ subunits of $G_\alpha$ by Western blotting. The data shown are representative of three to five independent experiments.

**Fig. 2. Binding of phosducin to $\alpha_\gamma$, and inhibition of this binding by PKA-mediated phosphorylation of phosducin.** The experiment was carried out as shown in Fig. 1B using 100 pmol of purified $\alpha_\gamma$ (instead of trimeric $G_\alpha$) and an equal amount of control or PKA-phosphorylated phosducin. No $\beta$ subunits could be detected in the $\alpha_\gamma$ preparation by Western blotting (not shown). The data are representative of three independent experiments.

**To confirm direct binding of $\alpha$ subunits to phosducin,** a second strategy involving another method to purify and to immobilize phosducin was used. To this end, a hexahistidine tag was added to the C terminus of phosducin, and phosducin-His$_6$ was purified to apparent homogeneity via binding of this hexahistidine tag to Ni-NTA-agarose. When 50 nM phosducin-His$_6$ were co-incubated with various concentrations of purified $G_{\beta\gamma}$ and then pelleted with Ni-NTA-agarose, $G_{\beta\gamma}$ was co-precipitated as detected by Western blots (Fig. 3A). Much less $G_{\beta\gamma}$ was precipitated in the absence of phosducin, indicating a relatively low nonspecific binding of $G_{\beta\gamma}$ to the resin. A semiquantitative analysis of this experiment by densitometry (Fig. 3B) revealed that the binding of $G_{\beta\gamma}$ to phosducin-His$_6$ was saturable with an apparent affinity of 25 nM. Analysis of four similar experiments gave an average apparent affinity of 55 ± 22 nM.

Analogous experiments were then done with purified $\alpha_\gamma$ (Fig. 4). Again, there was a phosducin-His$_6$-dependent precipitation of $\alpha_\gamma$ with Ni-NTA-agarose. A semiquantitative analysis showed saturable binding of $\alpha_\gamma$ to phosducin-His$_6$ with an ap-
trimeric Go. Phosducin inhibited the steady state GTPase ac-
tions (basal activity in 0.1% Lubrol), was inhibited by phos-
i.e. not only to the bg separable experiments.

Because G\textsubscript{o} and its subunits can be purified better than G\textsubscript{a} (and also because phosducin was initially discovered in the visual system) such experiments were also carried out with \alpha\textsubscript{o}, which had been purified to apparent homogeneity from bovine retina (Fig. 5). These experiments gave essentially similar results as those obtained with \alpha\textsubscript{a}, were required. The apparent affinity was 160 nM in the experiment shown in Fig. 5 and 200 ± 53 nM in a total of four separate experiments.

These data provide direct evidence for binding of phosducin not only to the \beta\gamma subunit complex but also to G-protein \alpha subunits. Functional assays were then used to determine the effects of such binding to \alpha subunits, again using \alpha\textsubscript{o}, because of its substantial intrinsic GTPase activity. Fig. 6 shows the effects of phosducin on the steady state GTPase activity of \alpha\textsubscript{o} and trimeric G\textsubscript{o}. Phosducin inhibited the steady state GTPase activity of \alpha\textsubscript{o} in a concentration-dependent manner; maximal inhibition was ~60%, and the IC\textsubscript{50} value was somewhat higher (~300 nM) than expected from the direct binding assays. As already shown earlier for trimeric G\textsubscript{o} (16) no such inhibition was seen with PKA-phosphorylated phosducin (data not shown). These results demonstrate a direct inhibitory effect of phosducin on the GTPase activity of \alpha\textsubscript{o} in agreement with its direct binding.

The GTPase activity of trimeric G\textsubscript{o} under the same conditions (i.e. basal activity in 0.1% Lubrol), was inhibited by phosducin up to ~90% with an IC\textsubscript{50} value of ~10 nM (Fig. 6). Assuming that this inhibition is caused by a direct effect of phosducin on \alpha\textsubscript{o}, and the well known effect via G\textsubscript{\gamma} we also analyzed this inhibition curve, assuming that it has a \beta\gamma-dependent and an \alpha-dependent component as described under

**Data Analysis.** This analysis gives a fit indicating that the \beta\gamma-dependent component causes 73% inhibition with an IC\textsubscript{50} value of ~7 nM, whereas the \alpha-dependent component causes an additional 19% inhibition with an IC\textsubscript{50} value of ~120 nM. The latter affinity is in the range of the IC\textsubscript{50} values determined
Fig. 6. Concentration-dependent inhibition $\alpha_c$ and $G_o$ GTPase activity by phosducin. The steady state GTPase activity of trimeric $G_o$ or isolated $\alpha_c$ (2 nM) in solution was assayed in the presence of the indicated concentrations of phosducin using an incubation time of 30 min and a reaction volume of 100 $\mu$l. Nonlinear curve fitting using the Hill equation gave the following estimates: $\alpha_c$: IC$_{50}$ 299 ± 26 nm; maximal inhibition, 61 ± 2%; $G_o$: IC$_{50}$ 10 ± 1 nm; maximal inhibition, 90 ± 1%. The latter set of data was fitted significantly better (dotted line, $p < 0.01$ by F-test) assuming a $\beta$-dependent and an $\alpha$-dependent component (see "Data Analysis") with the following estimates: $\beta$ component: IC$_{50}$, 7 ± 1 nm; maximal inhibition, 73 ± 3%; $\alpha$ component: IC$_{50}$ 120 ± 37 nm, maximal inhibition, 19 ± 3%. Data are the means ± S.E. from fourteen ($\alpha_c$) and eight ($G_o$) independent experiments.

from the direct effects of phosducin on $\alpha_c$; likewise, the extent of inhibition ($\sim$19/100–73), i.e., $\sim$70% is similar to the $\sim$60% inhibition seen in the GTPase assays with $\alpha_c$ alone. This two-component fit is statistically significantly better ($p < 0.01$ by F-test) than the simple one-component fit, but the differences between the two curves are very small.

The GTPase cycle contains two main steps: hydrolysis of GTP to GDP and release of GDP (followed by very rapid binding of GTP). The effects of phosducin on the first step in $\alpha_c$ were assayed in single-turnover experiments. In these assays, $[\gamma-32P]GTP$ is bound to $G_o$ in the absence of Mg$^{2+}$, conditions that suppress the enzymatic activity of G-proteins. The addition of Mg$^{2+}$ plus a large excess of a stable GTP analog then allows the monitoring of the hydrolysis of the bound $[\gamma-32P]GTP$. Fig. 7 shows that even a high concentration (2 $\mu$m) of phosducin had no appreciable effect on this catalytic step. GTP hydrolysis occurred with a rate constant of $4.8 \pm 0.4$ min$^{-1}$ in the absence and 3.9 ± 0.4 min$^{-1}$ in the presence of phosducin. The amount of $[\gamma-32P]GTP$ hydrolyzed was almost identical under the two conditions (0.51 ± 0.01 pmol/mg $G_o$ under control conditions versus 0.56 ± 0.02 in the presence of phosducin). These data indicate that the GTP-hydrolytic step of $G_o$ is not significantly affected by phosducin.

However, phosducin did impair the GDP release step of isolated $\alpha_c$. The presence of 0.5 $\mu$m phosducin reduced the rate constant of GDP release from isolated $\alpha_c$ from 0.23 ± 0.01 to 0.14 ± 0.01 min$^{-1}$ (Fig. 8). Concentration response curves for this inhibitory effect were done by monitoring the amount of GDP remaining bound to $\alpha_c$ 5 min after initiation of the release reaction (Fig. 9). Phosducin caused a concentration-dependent increase in the amount of bound GDP, and this effect was half-maximal at $\sim$100 nM. This concentration is in agreement with the apparent affinity of phosducin for $\alpha_c$ determined in the direct binding assays (Fig. 4B).

DISCUSSION

Phosducin is a widely distributed inhibitor of the GTPase activity of trimeric G-proteins (16, 17, 21, 22, 26). In addition to inhibition of the enzymatic activity of G-proteins themselves, it has also been shown to inhibit G-protein-mediated activation of adenyl cyclase in the $\beta$-adrenergic receptor system (16) and of cGMP phosphodiesterase in the rhodopsin system (17). In intact cells its overexpression impairs cAMP production induced by stimulation of $\beta$-adrenergic receptors (32). In the present study we have addressed the role of the G-protein subunits in this process and in particular whether in addition to the well demonstrated interactions with G-protein $\beta\gamma$ subunits phosducin might also have effects on the $\alpha$ subunits.

Our data indicate that phosducin can indeed interact directly with G-protein $\alpha$ subunits. This was shown in direct binding experiments as well as in functional assays. In the direct binding experiments two different factors were used to immobilize phosducin: specific affinity-purified antibodies and a C-terminal hexahistidine tag. These two strategies first permitted the use of two different initial purification procedures for phosducin and second should result in very different kinds of nonspecific binding of the G-protein subunits. With the antibody method no detectable amounts of G-protein subunits were re-
Fig. 9. Concentration-dependent inhibition of GDP release from isolated $G_o$ by phosducin. Shown is the amount of [32P]GDP remaining bound to $G_o$ (8 nM) 5 min after initiation of the release reaction as described in the legend to Fig. 8. Phosducin was present at the indicated concentrations. Nonlinear curve fitting gave an IC$_{50}$ value of 100 ± 17 nM. Data are the means ± S.E. from four independent experiments.

The apparent affinity of phosducin for $G_o$ determined in the direct binding assay was 110 nM, whereas in the functional assays IC$_{50}$ values of 100 nM (GDP release) and 300 nM (GTPase) were obtained; the analysis of the inhibition of $G_o$ GTPase with a $\beta\gamma$-mediated interaction and an $a$-dependent component gave an IC$_{50}$ value of the $a$-component of 120 nM. These affinities are obtained within the accuracy of the methods used.

The apparent affinity of phosducin for $G_o$ is lower than that for the $\beta\gamma$ subunit complex. However, an affinity in the range of 100–300 nM is still below the concentration of phosducin in most tissues, which is about 1 μM (21). Thus, this affinity is sufficient to allow phosducin-$G_o$ interactions in vivo. Likewise, the high concentrations of phosducin in the retina (18–21) are above the levels required for the low affinity interaction with $G_o$. Furthermore it appears that these interactions do indeed contribute to the inhibition of G-protein function by phosducin; the $G_o$ GTPase activity was inhibited by phosducin by up to 90% (Fig. 6). Under the conditions of these assays (in particular 0.1% Lubrol and high Mg$^{2+}$), $G_{\beta\gamma}$ causes a 2–4-fold activation of the GTPase activity of $G_o$ (39). Thus, if phosducin acted only to “trap” $G_{\beta\gamma}$, it should inhibit the GTPase activity of $G_o$ by at most 50–75%. This is clearly less than the observed inhibition of 90% and suggests an additional mode of inhibition. The analysis of this inhibition curve with two components ($a$ and $\beta\gamma$) did indeed result in a significantly better fit. Even though the improvement is only small, this two-component fit is entirely compatible in qualitative and in quantitative terms with the other data obtained here; it gives a $G_{\beta\gamma}$-mediated inhibition by 73% with an IC$_{50}$ value of 7 nM, compatible with the ranges given above, and a $G_o$-mediated inhibition of the remaining activity by 70% with an IC$_{50}$ value of 120 nM compatible with the affinities and the extent of inhibition seen in assays with isolated $G_o$.

The functional effects of phosducin on $G_o$ consist in an inhibition of GTP release but no effect on the catalytic step of the GTPase cycle. Thus, the effects of phosducin on $G_o$ appear to be composed of a direct inhibition of GDP release from $G_o$, and an antagonism of the effects of the $\beta\gamma$ subunit complex on the function of $G_o$. Because the $\beta\gamma$ subunits have stimulatory effects on $G_o$ under activating conditions, such as in the presence of high Mg$^{2+}$ concentrations, mastoparan, or active receptors (39), the direct and the $G_{\beta\gamma}$-mediated effects of phosducin on $G_o$ are additive under the conditions of stimulated G$_o$ activity.

The effects of phosducin on $G_o$ should not only cause an increased inhibition of G-protein function compared with $G_{\beta\gamma}$-mediated effects alone, but they should also increase the rapidity of this inhibition. This is because trapping of $G_{\beta\gamma}$ subsequent to G-protein activation would leave GTP-bound G$_o$ free to interact with effectors and disrupt the G-protein cycle only after the first round of GTP hydrolysis. In contrast, a direct effect on G$_o$ might already affect signaling in this first cycle of G-protein activation. Taken together these data suggest that the interactions of phosducin with $G_o$ are of functional relevance.

Direct interactions of phosducin with G-protein $a$ subunits were not observed by Lee et al. (17) and Yoshida et al. (26) in their investigations on transducin ($G_t$). These authors found no comigration of phosducin and $G_t$ on gel filtration columns and no effect of phosducin on the binding of $G_t$ to rod outer segment membranes. There are two possible explanations for this discrepancy. First, we found that the affinity of phosducin for $G_t$ is lower than that for $G_o$. Second, the assays used by these authors, which involve physical separation of proteins by chromatography or by centrifugation and washing, may be less sensitive to interactions of low affinity than the assays used here. In fact, gel filtration chromatography of phosducin has been shown to involve interactions with the matrix (26) that might well result in disruption of low affinity phosducin-$a$ subunit interactions, and the affinity of phosducin for $G_t$ reported in binding assays with rod outer segment membranes (26) is ~10-fold lower than that found in our assays.

In our hands, phosphorylation of phosducin by PKA impaired binding to $G_{\beta\gamma}$ as well as to $G_o$. This was seen both in direct binding and in GTPase assays. The data about the effect of phosphorylation on the phosducin/transducin-$\beta\gamma$ interaction are conflicting, depending on the assay used. Phosphorylated phosducin no longer coeluted with transducin-$\beta\gamma$ from gel filtration columns but still inhibited transducin-$\beta\gamma$ binding to rod outer segment membranes (26). It was concluded that phosphorylation might not alter the affinity of phosducin for transducin-$\beta\gamma$ but rather affect the character of the interaction (26). However, Hawes et al. (22) observed a loss of $G_{\beta\gamma}$ binding upon
phosphorylation of phosducin similar to our data. Because phosphorylation of phosducin would be required to impair Gbgγ binding by the C-terminal as well as the N-terminal binding sites, it is plausible to assume that it involves a major alteration of the structure of phosducin (24). Under these circumstances it is not surprising that phosphorylation of phosducin would also impair its interactions with Gα.

The molecular mechanisms of the interaction between phosducin and Gα remain to be elucidated. The crystal structure of phosducin complexed with the βγ subunits of Gα (24) suggests that phosducin binds with its C-terminal domain, particularly with an essential stretch of a few essential amino acids close to the C terminus (25), to the side of the β-propeller, whereas its less well defined N terminus is stretched out on the face of the propeller covering sites where Gbgα interacts with Gα. Detailed studies will be required to elucidate Gα-binding sites in phosducin. Furthermore, we do not know whether the interactions of phosducin with Gα involve two separate binding events (one with Gbgα and another one with αo), two-step binding (first to Gbgα and then to the αβγ trimer), or a single composite reaction to form a tetrameric phosducin-αβγ complex.

In summary, we believe that our data support interactions of phosducin with Gβγ as well as Gα. At low concentrations, phosducin appears to act preferentially by binding to Gβγ, and by neutralizing Gbgα activation. At higher concentrations, a direct inhibition of αo causes additional inhibition of G-protein function. Both types of interactions occur with affinities in the lower range of physiological phosducin concentrations in many tissues. Direct effects of phosducin on Gα are predicted to have two major effects compared with the previously presumed exclusive action via Gbgα; they would increase the extent as well as the rapidity of its inhibitory effects. Our observations suggest that complex interactions of phosducin with G-protein subunits play a role under physiological circumstances. These interactions provide an additional level to the many mechanisms (40) that regulate transmembrane signaling via G-protein-coupled receptors.

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Interactions of Phosducin with the Subunits of G-Proteins: BINDING TO THE \( \alpha \) AS WELL AS THE \( \beta\gamma \) SUBUNITS

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