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The Phosphorylation State of Phosducin Determines Its Ability to Block Transducin Subunit Interactions and Inhibit Transducin Binding to Activated Rhodopsin*

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Heterotrimeric GTP-binding proteins (G-proteins) serve many different signal transduction pathways. Phosducin, a 28-kDa phosphoprotein, is expressed in a variety of mammalian cell types and blocks activation of several classes of G-proteins. Phosphorylation of phosducin by cyclic AMP-dependent protein kinase prevents phosducin-mediated inhibition of G-protein GTPase activity (Bauer, P. H., Müller, S., Puzicha, M., Pippig, S., Obermaier, B., Helmreich, E. J. M., and Lohse, M. J. (1992) *Nature* 358, 73–76). In retinal rods, phosducin inhibits transducin (G_t) activation by binding its $\beta\gamma$ subunits. While rod phosducin is phosphorylated in the dark and dephosphorylated after illumination (Lee, R.-H., Brown, B. M., and Lolley, R. N. (1984) *Biochemistry* 23, 1972–1977), the significance of these reactions is still unclear. The data presented here permit a more precise characterization of phosducin function and the consequences of its phosphorylation.

Dephosphophosducin blocked binding of the $G_t\alpha$[1] subunit to activated rhodopsin in the presence of stoichiometric amounts of $G_t\beta\gamma$, whereas phosphophosducin did not. Surprisingly, the binding affinity of phosphophosducin for $G_t\beta\gamma$ was not significantly reduced compared with the binding affinity of dephosphophosducin. However, the association of phosducin with $G_t\beta\gamma$ in a size exclusion column matrix was dependent on the phosphorylation state of phosducin. Moreover, the ability of phosducin to compete with $G_t\alpha$ for binding to $G_t\beta\gamma$ was also dependent on the phosphorylation state of phosducin. No interaction was found between phosducin and $G_t\alpha$. These data indicate that phosducin decreases rod responsiveness by binding to the $\beta\gamma$ subunits of G_t and preventing their interaction with $G_t\alpha$, thereby inhibiting $G_t\alpha$ activation by the activated receptor. Moreover, phosphorylation of phosducin blocks its ability to compete with $G_t\alpha$ for binding to $G_t\beta\gamma$.

Heterotrimeric G-proteins comprise a gene family whose members function in a remarkable variety of signal transduction pathways. Binding of G-proteins to their activated receptors results in exchange of GDP for GTP and dissociation of the GTP-bound $G\alpha$ subunit from its $G\beta\gamma$ subunits. Activated $G\alpha$s ($G\alpha$·GTP's) regulate a variety of effector protein systems, including cyclic nucleotide processing enzymes, phospholipases, and cation-selective channel proteins (for review, see Refs. 1–3). In addition, $G\beta\gamma$ subunits have recently been shown to regulate directly effectors such as phospholipase A_2 (4), β-adrenergic receptor kinase (5), type II adenylyl cyclase (6, 7) and phospholipase C-β (8, 9). GTP hydrolysis by $G\alpha$ allows the reassociation of $G\alpha$·GDP with $G\beta\gamma$, returning the system to its ground state in which $G\alpha\beta\gamma$·GDP is again capable of binding to an activated receptor.

Phosducin is a highly conserved 28-kDa phosphoprotein that is expressed in a number of cell types including retinal rods and cones, pinealocytes, neurons, hepatocytes, and myocytes (10–13). Moreover, the retinal protein MEKA was found to be virtually identical to phosducin from retina and brain (11, 12, 14). Phosducin inhibits the GTPase activities of a number of G-proteins including G_i, G_s, and G_o (12); phosphorylation of phosducin with cyclic AMP-dependent protein kinase prevents this GTPase inhibition (12). Phosducin was first isolated in vertebrate retinal rod cells in a complex with $G_t\beta\gamma$ subunits (15), and it has been proposed that phosducin interferes with transducin (G_t) function by binding to $G_t\beta\gamma$ (16). While rod phosducin is known to be phosphorylated in the dark and dephosphorylated after illumination (17), the functional significance and biochemical details of these phosphorylation and dephosphorylation reactions have not been well defined. Here we report new observations that permit a more precise characterization of the functional biochemistry of rod phosducin and the consequences of its phosphorylation.

EXPERIMENTAL PROCEDURES

Preparation of Bovine Rod Outer Segments —Rod outer segments (ROS) were prepared by sucrose floatation (18). All manipulations of dark-adapted retinas were performed under infrared illumination using infrared image converters. Briefly, 250 fresh dark-adapted bovine retinas (J.A. & W.L. Lawson Co., Lincoln, NB) were suspended in the dark in 400 ml of HEPES/Ringer's buffer (10 mM HEPES pH 7.5, 120 mM NaCl, 3.5 mM KCl, 0.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 0.1 mM EDTA, 10 mM glucose, 1 mM DTT, 0.2 mM PMSF) plus 45% (w/w) sucrose and disrupted by being drawn twice through a 60-ml syringe with a 0.7-cm diameter orifice. The disrupted retinas were passed through two layers of cheesecloth, and the retinal material that did not pass through the cheesecloth was supended in 50 ml of HEPES/Ringer's buffer, drawn through the syringe again, and reapplied to the cheesecloth. This process was repeated three times. The retinal material that did not pass through the cheesecloth was used for phosducin purification. The filtrate from the cheesecloth was centrifuged for 10 min at 4,000 × *g*. The resulting pellet was used for phosducin purification, and the supernatant, containing the ROS, was diluted to ~15% sucrose with HEPES/Ringer's buffer.

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[1] The abbreviations used are: G_t, retinal rod G-protein, transducin; Rho, rhodopsin; Rho*, light-activated rhodopsin; ROS, retinal rod outer segment, PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl flouride.

This suspension was centrifuged at 10,000 × *g* for 20 min. The resulting supernatant was used for phosducin purification, and the pellet was suspended in HEPES/Ringer's buffer, layered over 15 ml of 33% (w/w) sucrose in 6–8 tubes, and centrifuged at 40,000 × *g* for 15 min. The ROS were collected from the top of the 33% sucrose layer, diluted to ~15% sucrose with HEPES/Ringer's, and centrifuged at 25,000 × *g* for 10 min. From these ROS, G_t subunits and urea-stripped ROS membranes were prepared.

Protein Purifications and Membrane Preparations—$G_t\alpha$, $G_t\beta\gamma$, and urea-stripped ROS membranes were prepared as described (19) except that after separation of G_t subunits on a Blue Sepharose column (Pharmacia Biotech Inc.), the subunits were further purified with a high performance liquid chromatography (HPLC) size exclusion column (Bio-Silect SEC-250, Bio-Rad) by isocratic elution in 10 mM HEPES pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF (buffer A). The resulting $G_t\beta\gamma$ was >98% pure and was free of $G_t\alpha$ as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE). The $G_t\alpha$ was >95% pure and was likewise free of $G_t\beta\gamma$ subunits. $G_t\alpha$ and $G_t\beta\gamma$ were labeled with ^{125}I using Bolton-Hunter reagent (DuPont NEN) as described previously (19).

Phosducin was purified by a modification of previously described procedures (11, 15). The retinal debris retained on the cheesecloth was released and homogenized in 5 mM Tris·Cl, pH 7.5, 62 mM NaCl, 5 mM $MgCl_2$, 2 μM leupeptin, and 2 μM pepstatin A in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 48,000 × *g* for 10 min; the supernatant was saved and the pellet was homogenized once more. The pellet from the 45% sucrose centrifugation step above was also homogenized twice. The supernatants were combined with the supernatant from the 15% sucrose centrifugation step (~2 liters), and proteins were precipitated by the addition of 474 g/liter ammonium sulfate. The solution was centrifuged at 30,000 × *g* for 25 min, and the pellet was resuspended in 30 mM potassium phosphate, pH 7.0, 1 mM DTT, 1 mM EDTA, and 0.1 mM PMSF (buffer B). The suspension was dialyzed overnight against 9 liters of buffer B with one change of buffer after 6 h. The suspension was adjusted to pH 5.7 with 1 M acetic acid and centrifuged at 48,000 × *g* for 20 min. The pH and the conductivity of the solution were adjusted to 7.0 and 6 millisiemens/cm, respectively, and loaded at 4 ml/min at 4 °C on TSK-Gel DEAE Toyopearl 650 M (TosoHaas) column material packed in an HR16/50 column (Pharmacia), using a Pharmacia fast protein liquid chromatography system. After the unbound material was washed through with buffer B, phosducin was eluted with 600 ml of a linear gradient from 30–500 mM potassium phosphate in buffer B at a flow rate of 4.5 ml/min. The fractions with conductivities between 13 and 31 millisiemens/cm were pooled and dialyzed against 10 mM potassium phosphate, pH 7.0, 1 mM DTT, and 0.1 mM PMSF (buffer C). The solution was loaded onto Macro-Prep ceramic hydroxyapatite (40 μm, Bio-Rad) column material packed in an XK26/40 column (Pharmacia) equilibrated with the buffer C using fast protein liquid chromatography at a flow rate of 3 ml/min at 4 °C. The phosducin was eluted with 300 ml of a linear gradient of buffer C to buffer B containing 150 mM potassium phosphate, pH 7.0, at 3 ml/min. Fractions with conductivities of 6–14 millisiemens/cm were pooled and concentrated to ~1.5 ml with CentriPrep10 (Amicon) concentrators. The concentrated phosducin fraction was chromatographed on a TSK G3000SW size exclusion column (TosoHaas, 21.5 mm (inner diameter) × 30 cm with a 21.5 mm inner diameter × 7.5 cm guard column) running isocratically with buffer A at 5 ml/min using a Waters 625LC HPLC system at 23 °C. The phosducin-$G_t\beta\gamma$ complex eluted at 18 min. Phosducin was separated from $G_t\beta\gamma$ and other contaminants by strong anion exchange chromatography using a Poros Q/M 4.6/100 column (PerSeptive Biosystems). The column was equilibrated in 50 mM Tris, pH 8.0, 0.1 mM PMSF, and 1 mM DTT and was eluted by a linear gradient of 50–750 mM Tris at 3 ml/min (30 ml). The phosducin eluted at ~560 mM Tris. Phosducin was further purifed by sucrose density gradient centrifugation (5 ml of 5–20% sucrose, 100 mM potassium phosphate, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and ~ 0.5 mg of protein/tube) in a Beckman SW 55Ti rotor running in a Beckman L8–70 M centrifuge for 15.3 h at 53,000 rpm ($\omega^2 t = 1.7 \times 10^{12}$ rad^2/s) at 4 °C. This phosducin was 95% pure and was free of $G_t\beta\gamma$ as determined by SDS-PAGE and Western blotting with a peptide-specific antibody for $G_t\beta$. Protein concentrations were determined using Coomassie Plus protein assay reagent (Pierce Chemical Co.).

Phosducin Phosphorylation and Isoelectric Focusing—Phosducin was phosphorylated with the catalytic subunit of bovine heart cAMP-dependent protein kinase (Fluka) as follows. Phosducin (30 μM) was mixed with cAMP-dependent protein kinase (1 unit/μl) and ATP (0.5 mM) in 50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF and incubated for 10 min at 23 °C. Phosphophosducin was immediately assayed for ^{125}I-$G_t\alpha$ binding (see below). For isoelectric focusing, phosducin was phosphorylated as described above in the presence of 0.5 mM (~1000 dpm/pmol) [^{33}P]ATP, and then focused on a flat bed isoelectric focusing gel (0.4 mm) according to the manufacturer's (Bio-Rad) protocol using Pharmalyte 4.5–5.4 ampholines (Pharmacia) in the presence of 8 M urea. The gel was fixed in 20% trichloroacetic acid for 30 min. Ampholines were eluted from the gel by incubating in 0.25% SDS, 10% acetic acid, and 40% methanol for 30 min followed by 10% acetic acid and 40% methanol for 1 h. The gel was stained with 0.12% Coomassie Brilliant Blue R (Serva) in 10% acetic acid and 40% methanol for 30 min and then destained in the same solution without Coomassie Blue. Finally, the gel was autoradiographed to verify the incorporation of phosphate into phosducin.

Binding of ^{125}I-$G_t\alpha$ to Rho in ROS Membranes*—Light-induced binding of ^{125}I-$G_t\alpha$ and $G_t\beta\gamma$ to urea-stripped ROS membranes was carried out as described (19). Dephospho- or phosphophosducin, at the concentrations indicated, was mixed with 0.1 μM ^{125}I-$G_t\alpha$ and 0.1 μM $G_t\beta\gamma$ before the addition of urea-stripped membranes in the dark. Light-induced binding was then initiated by bleaching 50% of the Rho. Samples were centrifuged, and the amount of ^{125}I-$G_t\alpha$ bound was quantified as described (19).

Phosducin Inhibition of ^{125}I-$G_t\beta\gamma$ Binding to ROS Membranes—The effect of phosducin on the binding of ^{125}I-$G_t\beta\gamma$ to urea-stripped ROS membranes was measured by incubating 0.35 μM ^{125}I-$G_t\beta\gamma$ with increasing concentrations of phosducin in isotonic buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 20 mM NaCl, 1.5 mM $CaCl_2$, 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF) for 10 min at 23 °C. Unilluminated urea-stripped membranes, at 40 μM Rho, were then added in the dark, and the mixture was incubated in the dark for 10 min. The final sample volume was 50 μl. For phosphophosducin, 0.5 units/μl of cAMP-dependent protein kinase, and 0.5 mM ATP were added to phosducin in isotonic buffer, and phosphorylation was allowed to continue for 5 min before the addition of the ^{125}I-$G_t\beta\gamma$ and dark urea-stripped membranes. During the dark incubation, 20 μl of the sample were taken and counted for ^{125}I to obtain the total counts. The samples were then centrifuged at 50,000 × *g* for 20 min to pellet the membranes, and 20 μl of the supernatant were removed and counted for ^{125}I. The amount of ^{125}I-$G_t\beta\gamma$ in the membrane pellet was calculated by subtracting the supernatant counts from the total counts in the sample. During these experiments, the phosphorylation state of phosducin was monitored in parallel samples that were incubated with 0.5 mM [^{32}P]ATP (500 cpm/pmol). Incorporation of ^{32}P into phosducin was quantified by precipitating 10 μl of the sample in 100 μl of 20% trichloroacetic acid, filtering through type HA nitrocellulose filters in 96-well Multiscreen filtration plates (Millipore), washing three times with 100 μl of 20% trichloroacetic acid, and counting the ^{32}P on the filter. Phosducin was completely phosphorylated by 2 min and remained phosphorylated throughout the experiment. Control samples without phosducin were run in order to correct for phosphorylation of the urea-stripped membranes under these conditions.

In order to obtain a binding constant for the interaction between ^{125}I-$G_t\beta\gamma$ and phosducin, an equation for the binding of ^{125}I-$G_t\beta\gamma$ to urea-stripped ROS membranes as a function of the total concentration of phosducin was derived from the following model (Equation 1), which describes in simplest terms the binding of ^{125}I-$G_t\beta\gamma$ to the membrane

$$\begin{array}{ccc} & & K_{d1} \\ ^{125}\text{I-G}_t\beta\gamma + \text{M} & \rightleftharpoons & \text{M}\cdot{}^{125}\text{I-G}_t\beta\gamma \\ + & & \\ \text{Pd} & & \\ \Updownarrow K_{d2} & & \\ \text{Pd}\cdot{}^{125}\text{I-G}_t\beta\gamma & & \end{array} \qquad \text{(Eq. 1)}$$

where M is the membrane binding site specific for ^{125}I-$G_t\beta\gamma$, and Pd is phosducin. From the expressions for the dissociation constants,

$$K_{d1} = [M][^{125}\text{I} - \text{G}_t\beta\gamma]/[M\cdot{}^{125}\text{I-G}_t\beta\gamma] \qquad \text{(Eq. 2)}$$

$$K_{d2} = [\text{Pd}][^{125}\text{I} - \text{G}_t\beta\gamma]/[\text{Pd}\cdot{}^{125}\text{I-G}_t\beta\gamma] \qquad \text{(Eq. 3)}$$

and the expressions for the conservation of mass,

$$[M]_t = [M\cdot{}^{125}\text{I-G}_t\beta\gamma] + [M] \qquad \text{(Eq. 4)}$$

$$[\text{Pd}]_t = [\text{Pd}\cdot{}^{125}\text{I-G}_t\beta\gamma] + [\text{Pd}] \qquad \text{(Eq. 5)}$$

$$[^{125}\text{I-G}_t\beta\gamma]_t = [M\cdot{}^{125}\text{I-G}_t\beta\gamma] + [\text{Pd}\cdot{}^{125}\text{I-G}_t\beta\gamma] + [^{125}\text{I-G}_t\beta\gamma] \qquad \text{(Eq. 6)}$$

an expression for $[M\cdot{}^{125}\text{I-G}_t\beta\gamma]$ was derived in the form of a cubic equation.

$A[M\cdot^{125}I\text{-}G_t\beta\gamma]^3 + B[M\cdot^{125}I\text{-}G_t\beta\gamma]^2 + C[M\cdot^{125}I\text{-}G_t\beta\gamma] + D = 0$, where

$$A = K_{d2} - K_{d1}$$

$$B = K_{d1}^2 - K_{d1}K_{d2} - K_{d1}[Pd]_t + K_{d1}[M]_t - 2K_{d2}[M]_t + K_{d1}[^{125}I\text{-}G_t\beta\gamma]_t - K_{d2}[^{125}I\text{-}G_t\beta\gamma]_t$$

$$C = K_{d1}K_{d2}[M]_t + K_{d1}[Pd]_t[M]_t + K_{d2}[M]_t^2 - K_{d1}[^{125}I\text{-}G_t\beta\gamma]_t[M]_t + 2K_{d2}[^{125}I\text{-}G_t\beta\gamma]_t[M]_t$$

$$D = -K_{d2}[^{125}I\text{-}G_t\beta\gamma]_t[M]_t^2$$

The equation was solved numerically by Newton's method and was used to curve fit the data for the binding of $^{125}I\text{-}G_t\beta\gamma$ to membranes in the presence of phosducin. The values of $[^{125}I\text{-}G_t\beta\gamma]_t$ (0.35 μM) and $[Pd]_t$ (varied from 0–4.8 μM) were known from the experimental conditions, and initial values of K_{d1} (2.0 μM) and $[M]_t$ (2.4 μM) were obtained in separate $^{125}I\text{-}G_t\beta\gamma$ binding experiments in the absence of phosducin. The curve fits of the data yielded best-fit values for K_{d1}, K_{d2}, and $[M]_t$. The experimental data showed saturation values that deviated from zero by ~8% (see Fig. 4*A*) due to nonspecific interactions of $^{125}I\text{-}G_t\beta\gamma$ with the membrane or low affinity binding of phosducin-$^{125}I\text{-}G_t\beta\gamma$ to the membrane. In order for Equation 7 to predict saturation at $[M\cdot^{125}I\text{-}G_t\beta\gamma]$ values greater than zero, $[M]_t$ was defined as: $[M]_t = [M]_{max} - [M]_{min}$, where $[M]_{max}$ is the total concentration of membrane binding sites and $[M]_{min}$ is the concentration of nonspecific binding sites.

Effect of $G_t\alpha$ on Phosducin Inhibition of $^{125}I\text{-}G_t\beta\gamma$ Binding to ROS Membranes—In the same experimental format used in the previous section, 2 μM phosducin was added to increasing concentrations of $G_t\alpha$ followed by 0.35 μM $^{125}I\text{-}G_t\beta\gamma$, and then unilluminated urea-stripped ROS (40 μM Rho) were added in the dark. The fraction of $^{125}I\text{-}G_t\beta\gamma$ in the supernatant and the extent of phosducin phosphorylation were quantified as described above.

Effect of Phosducin on $^{125}I\text{-}G_t\alpha$ Binding to ROS Membranes—In a similar experimental format, increasing concentrations of phosducin were added to 0.35 μM $^{125}I\text{-}G_t\alpha$ followed by 40 μM Rho in heat-denatured urea-stripped ROS membranes. The urea-stripped membranes were heat denaturated at 70 °C for 5 min prior to the addition of $^{125}I\text{-}G_t\alpha$ and phosducin to remove residual amounts of $G_t\beta\gamma$ from the membranes (see "Results").

Gel Analysis of $G_t\alpha$, $G_t\beta\gamma$ and Phosducin Binding to ROS Membranes—Phosducin (4 μM) was mixed with $G_t\beta\gamma$ (6 μM) in the presence or absence of $G_t\alpha$ (16 μM) in isotonic buffer. The mixture was incubated with unilluminated urea-stripped ROS membranes (40 μM Rho) for 10 min in the dark at 23 °C. The samples were centrifuged at 50,000 × *g* for 20 min. The membrane pellets were resuspended in the dark with 500 μl of hypotonic buffer (10 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.2 mM PMSF) and incubated for 30 min at 23 °C. The suspension was again centrifuged at 50,000 × *g* for 20 min, and the resulting supernatant was concentrated to 35 μl using Microcon 10 (Amicon) concentrators. 15 μl of the concentrated supernatant were analyzed by SDS-PAGE on a 12% Laemmli gel (20). The gel was stained with 0.12% Coomassie Brilliant Blue R (Serva) in 10% acetic acid and 40% methanol for 30 min and then destained in the same solution without Coomassie Blue.

HPLC Size Exclusion Chromatography—A Bio-Silect SEC 250–5 (Bio-Rad) size exclusion column was used for analytical HPLC analysis. The column matrix consists of a silica-based gel. Samples containing phosducin, phosphophosducin, $G_t\beta\gamma$ and $G_t\alpha$ (as indicated in the figure legends) were injected into the column and eluted isocratically with buffer A at a flow rate of 1.0 ml/min. Protein peaks were detected by measuring the absorbance of the eluate at 254 nm. Fractions from the column were pooled as indicated and concentrated in Centricon 10 (Amicon) microconcentrators, and equivalent volumes were analyzed by 12% SDS-PAGE (20). For phosducin plus $G_t\beta\gamma$ samples, the proteins were mixed and incubated for 5 min at 23 °C before injection. Phosphophosducin plus $G_t\beta\gamma$ was prepared by phosphorylating phosducin as described above and then adding $G_t\beta\gamma$ and incubating for 5 min at 23 °C before injection into the column. The same incubation steps were followed in assessing $G_t\alpha$ binding to phosducin.

RESULTS

Phosphorylation State-dependent Inhibition of $^{125}I\text{-}G_t\alpha$ Binding to Rho by Phosducin*

In an effort to understand the mechanism of phosducin inhibition of G-protein activation and to assess the consequences of phosducin phosphorylation, direct effects of phosducin on $^{125}I\text{-}G_t\alpha$ binding to Rho* were measured. Phosducin or phosphophosducin was added in increasing concentrations to $^{125}I\text{-}G_t\alpha$, $G_t\beta\gamma$, and urea-stripped ROS membranes in $^{125}I\text{-}G_t\alpha$ binding assays (19). The binding of $^{125}I\text{-}G_t\alpha$ to Rho* was prevented by phosducin (Fig. 1*A*). At $^{125}I\text{-}G_t\alpha$ and $G_t\beta\gamma$ concentrations of 0.1 μM, half-maximal inhibition occurred at ~0.3 μM phosducin, and binding was totally prevented at 2 μM phosducin. Inhibition was cooperative (n_{app} = 1.6 ± 0.2), consistent with the cooperative nature of G_t binding to Rho* (19) and indicating that phosducin does control the concentration of free $G_t\alpha\beta\gamma$. In light of these data, the observed inhibition of G-protein GTPase activity and effector enzyme activation by phosducin (12, 16) is best explained by phosducin's blocking G-protein interaction with the activated receptor.

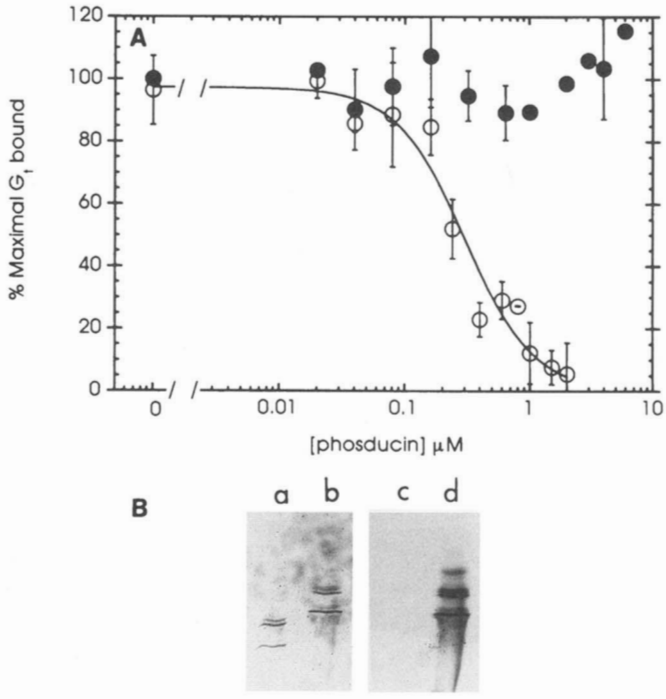

FIG. 1. **Phosphorylation of phosducin regulates its ability to inhibit $^{125}I\text{-}G_t\alpha$ binding to light-activated rhodopsin.** *A*, light-induced binding of $^{125}I\text{-}G_t\alpha$ (0.1 μM) and $G_t\beta\gamma$ (0.1 μM) to urea-stripped ROS membranes (0.2 μM Rho) was carried out in the presence of unphosphorylated (○) or phosphorylated (●) phosducin at the concentrations indicated (see "Experimental Procedures"). *Error bars* represent the standard error of data from three separate experiments. 100% is 0.17–0.20 pmol of $^{125}I\text{-}G_t\alpha$ bound/pmol of Rho*. The *solid line* represents a curve fit of the data to the following equation. % bound = 100/(1+([phosducin]/$K_{½}$)^n); where $K_{½}$ = 0.3 ± 0.05 μM and n = 1.6 ± 0.2. The phosphorylation mixture without phosducin had no effect on $^{125}I\text{-}G_t$ binding (data not shown). *B*, phosducin was phosphorylated and isoeletric focusing was performed as described under "Experimental Procedures." *Lane a*, Coomassie Blue stain of unphosphorylated phosducin; *lane b*, similar stain of phosphorylated phosducin; *lanes c* and *d*, corresponding autoradiograph of unphosphorylated and phosphorylated phosducin, respectively.

When phosducin was phosphorylated with cAMP-dependent protein kinase and ATP before assaying for inhibiton of $^{125}I\text{-}G_t\alpha$ binding to Rho*, no inhibition was found even at phoducin concentrations up to 6 μM (Fig. 1*A*). This corresponds to a decrease of at least 50-fold in phosphophosducin's ability to inhibit $^{125}I\text{-}G_t\alpha$ binding compared with that of dephosphophosducin.

The extent of phosducin phosphorylation under these conditions was determined by isoelectric focusing (Fig. 1*B*). In isoelectric focusing gels, three major isoforms of phosducin were observed near pI 5.2. These isoforms may result from sequence variations in phosducin. In retinal rods, variants of phosducin at amino acid position 44 have been reported. One isoform contains a His residue (11, 12, 21), whereas the second isoform

contains a Pro residue (10, 12). The different isoforms could also result from modifications during purification such as proteolytic cleavage. However, if proteolysis had been occurring, it could not have been extensive because no proteolytic products were observed by SDS-PAGE, which could detect the loss of fragments of 1 kDa or larger. Phosphorylation by cAMP-dependent protein kinase shifted all isoforms to more acidic pI values (compare *lanes a* and *b*) and an autoradiograph of the gel (*lanes c* and *d*) showed that all isoforms were phosphorylated (*lane d*). Moreover, no residual phosducin was detected in the bands corresponding to unphosphorylated phosducin. Thus, it appears that none of the isoforms are phosphorylated in the purified phosducin and that all isoforms are completely phosphorylated upon treatment with cAMP-dependent protein kinase and ATP.

The data presented in Fig. 1 indicate that phosducin's ability to inhibit ^{125}I-$G_t\alpha$ binding to Rho* is decreased more than 50-fold when phosducin is in its phosphorylated state.

Dephospho- and Phosphophosducin Binding to $G_t\beta\gamma$—Phosducin is known to interact with $G_t\beta\gamma$ and appears to dissociate solubilized $G_t\alpha\beta\gamma$ (16). Since $G_t\beta\gamma$ is required for high affinity binding of $G_t\alpha$ to Rho* (22–24), phosducin could block $G_t\alpha$ binding to Rho* by sequestering $G_t\beta\gamma$ in a phosducin-$G_t\beta\gamma$ complex. If this is the case, then phosphorylation of phosducin should inhibit phosducin-$G_t\beta\gamma$ interactions, since phosphophosducin did not block ^{125}I-$G_t\alpha/G_t\beta\gamma$ binding to Rho*. The effect of phosducin phosphorylation on the interaction with $G_t\beta\gamma$ was directly examined in a silica gel-based HPLC size exclusion column. Unmodified phosducin eluted from an SEC 250–5 column with a retention time of 8.4 min, which was earlier than the predicted retention time for a 28-kDa protein (9.6 min, Fig. 2, *A* and *E*). This early elution could result from phosducin oligomerization or electrostatic repulsion of phosducin by the column matrix. The latter possibility appears likely because increasing the NaCl concentration from 0.5 to 1.0 M increased the retention of phosducin on the column to near normal for a 28-kDa monomer. Furthermore, sucrose density gradient centrifugation analysis of phosducin gave no apparent evidence for an oligomer. Using bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* as standards, phosducin exhibited an $s_{20,w}$ value of 2.2 S, which is somewhat lower than expected for a globular protein of 28 kDa.

$G_t\beta\gamma$ also exhibited an unexpected elution rate from the column. It was much delayed and eluted at 11.8 min, later than the total column volume (Fig. 2, *B* and *E*). This retention of $G_t\beta\gamma$ on the column seems to be a result of hydrophobic interactions with the column matrix, since increasing the NaCl concentration further increased the retention time of $G_t\beta\gamma$. These unusual interactions of phosducin and $G_t\beta\gamma$ with the column matrix were exploited to obtain excellent separation of phosducin and $G_t\beta\gamma$ on the column. When $G_t\beta\gamma$ was incubated with phosducin prior to sample injection, $G_t\beta\gamma$ eluted at 8.4 min, overlapping the phosducin peak. This is evidenced by an increase in the area under the phosducin peak, by a shifting of $G_t\beta\gamma$ into this peak (Fig. 2*F*, *lane c*), and by the disappearance of $G_t\beta\gamma$ at the 11.8 min elution time (Fig. 2, *C* and *F*, *lane e*). Thus, binding to phosducin caused $G_t\beta\gamma$ no longer to interact with the column matrix but to elute in a complex with phosducin that exhibited a retention time similar to that of free phosducin. This corresponds to the expected retention time for a $G_t\beta\gamma$·phosducin oligomer of 74 kDa. A very small fraction of the $G_t\beta\gamma$ was found at elution times between that of phosducin-$G_t\beta\gamma$ and free $G_t\beta\gamma$ (Fig. 2*F*, *lane d*), suggesting that the phosducin-$G_t\beta\gamma$ complex is quite stable on the column and that the column matrix is not slowly removing $G_t\beta\gamma$ from the phosducin-$G_t\beta\gamma$ complex as it passes through the column.

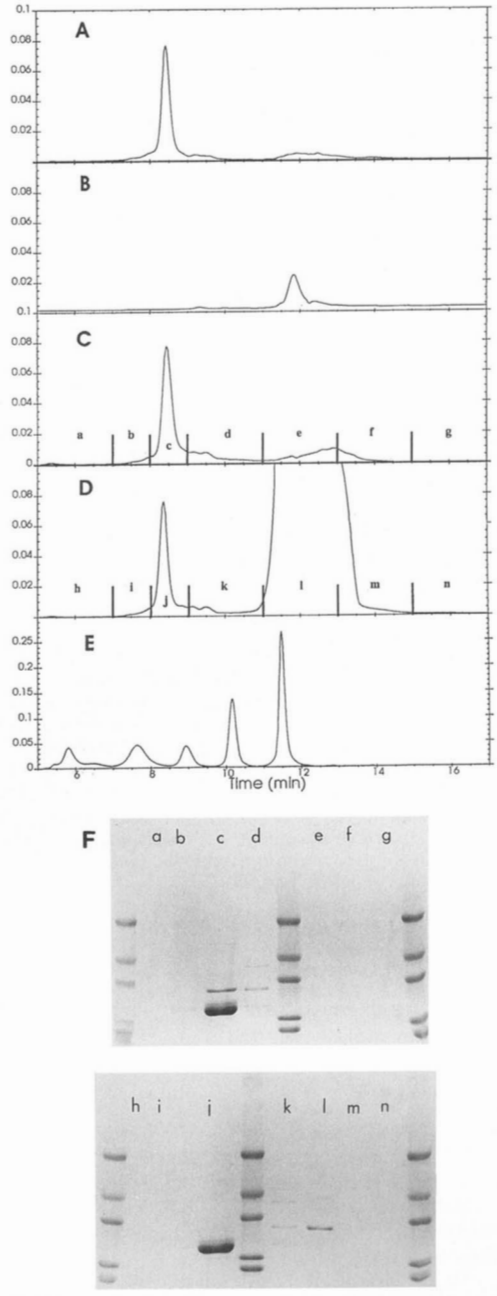

FIG. 2. **HPLC analysis of interactions of phosducin and $G_t\beta\gamma$.** HPLC size exclusion chromatography and gel analysis of peak fractions were performed as described under "Experimental Procedures." Elution profiles from the size exclusion column are shown as follows: *A*, 3.3 nmol of phosducin; *B*, 1.1 nmol of $G_t\beta\gamma$; *C*, 3.3 nmol of phosducin and 1.1 nmol $G_t\beta\gamma$; *D*, 3.3 nmol of phosphophosducin, 1.1 nmol of $G_t\beta\gamma$, 200 units of cAMP-dependent protein kinase, 12 nmol of ATP; *E*, molecular mass standards with the following retention times: 5.8 min, bovine thyroglobulin (670 kDa); 7.6 min, bovine γ globulin (158 kDa); 8.9 min, chicken ovalbumin (44 kDa); 10.2 min, horse myoglobin (17 kDa); and 11.5 min, vitamin B-12 (1.35 kDa). *F*, SDS-PAGE of fractions (*a–n*) from the chromatograms as indicated. In *panel C*, phosducin and $G_t\beta\gamma$ were incubated for 5 min at 23 °C prior to sample injection. In *panel D*, phosphophosducin was prepared according to "Experimental Procedures," and then $G_t\beta\gamma$ was added and incubated for 5 min at 23 °C prior to sample injection. SDS-PAGE molecular mass standards are shown and correspond to the following molecular masses from *top* to *bottom*: 66, 45, 36, 29, and 24 kDa.

Next, the interaction between phosphophosducin and $G_t\beta\gamma$ was examined in the presence of cAMP-dependent protein kinase and ATP, which were present to assure persistent phosducin phosphorylation. (Phosphophosducin was found to be ex-

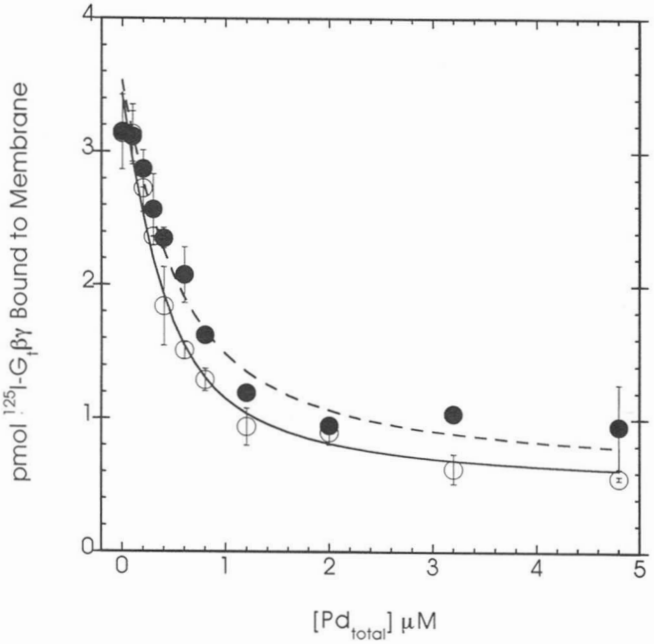

FIG. 3. **Phosducin inhibition of ^{125}I-G$_t\beta\gamma$ binding to ROS membranes.** ^{125}I-G$_t\beta\gamma$ (0.35 μM) binding to unilluminated urea-stripped ROS membranes (40 μM, Rho) was measured at increasing concentrations of phosducin (○) or phosphophosducin (●) as indicated (see "Experimental Procedures"). The total ^{125}I-G$_t\beta\gamma$ in the 20-μl sample was 7.0 pmol. *Error bars* represent the standard error of the data from three separate experiments. The data were curve fit as described under "Experimental Procedures," yielding a K_{d2} value of 0.11 μM for phosducin binding to ^{125}I-G$_t\beta\gamma$ and 0.18 μM for phosphophosducin binding to ^{125}I-G$_t\beta\gamma$. The saturation points for the inhibition were at 0.48 and 0.58 pmol for dephospho- and phosphophosducin, respectively. The curve fitted value of K_{d1} for the binding of ^{125}I-G$_t\beta\gamma$ to the urea-stripped membranes was 2.2 μM for dephosphophosducin, and this value was fixed at 2.2 μM in fitting the phosphophosducin curve.

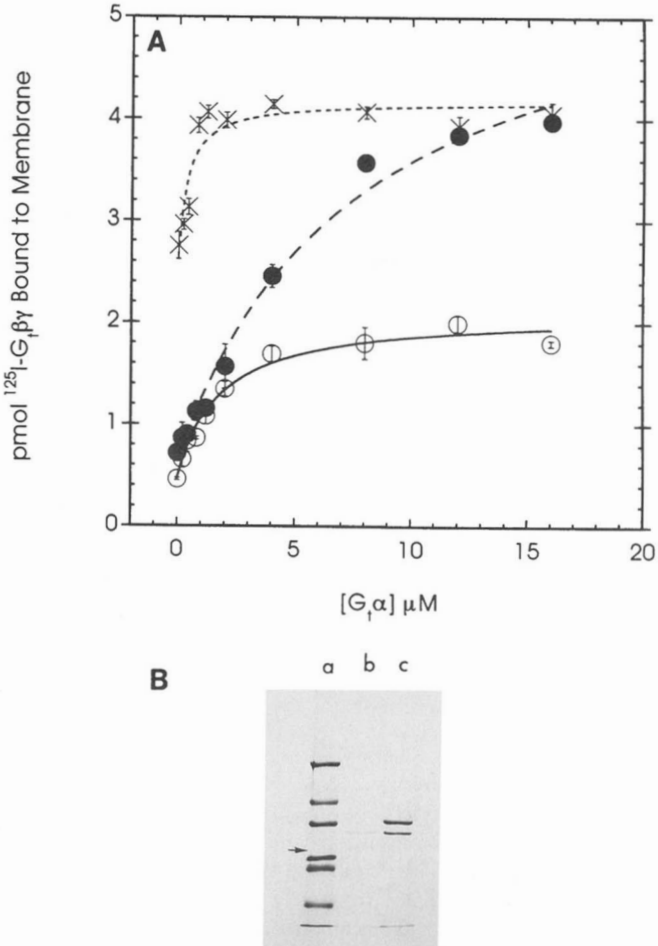

FIG. 4. **G$_t\alpha$ competition with phosducin for ^{125}I-G$_t\beta\gamma$ binding.** *A*, binding of ^{125}I-G$_t\beta\gamma$ (0.35 μM) to unilluminated urea-stripped ROS membranes (40 μM Rho) was measured in the presence (○) and absence (×) of phosducin (2.0 μM) or phosphophosducin (●) at the concentrations of G$_t\alpha$ indicated (see "Experimental Procedures"). The total ^{125}I-G$_t\beta\gamma$ in the 20-μl sample was 7.0 pmol. *Error bars* represent the standard error of the data from two separate experiments, and these data are representative of data obtained from three different phosducin preparations. The data were fit to the following equation. ^{125}I-G$_t\beta\gamma$ bound = ((^{125}I-G$_t\beta\gamma$ max − ^{125}I-G$_t\beta\gamma$ min) [G$_t\alpha$]/($K_{½}$ + [G$_t\alpha$])) + G$_t\beta\gamma$ min; where ^{125}I-G$_t\beta\gamma$ max is the amount bound at saturating G$_t\alpha$ and ^{125}I-G$_t\beta\gamma$ min is the amount bound in the absence of G$_t\alpha$. Fitting the data in this manner resulted in $K_{½}$ = 0.39 ± 0.2 μM in the absence of phosducin, $K_{½}$ = 1.8 ± 0.4 μM in the presence of dephosphophosducin, and $K_{½}$ = 7.5 ± 1.9 μM in the presence of phosphophosducin. *B*, SDS-PAGE analysis of the binding of G$_t\alpha$, G$_t\beta\gamma$, and phosducin to urea-stripped ROS membranes was performed as described under "Experimental Procedures" with 4 μM phosducin, 6 μM G$_t\beta\gamma$, and 16 μM G$_t\alpha$. Hypotonic extracts of the membrane pellet in the presence (*lane c*) or the absence (*lane b*) of G$_t\alpha$ are shown. The *arrow* indicates the migration position for phosducin. Molecular weight standards (*lane a*) are from *top* to *bottom*: 66, 45, 36, 29, 24, and 20 kDa.

tremely sensitive to trace amounts of phosphatase activity.) After a phosducin sample was phosphorylated, G$_t\beta\gamma$ was added, and the mixture was injected. In this case, the phosducin peak remained unchanged, and the G$_t\beta\gamma$ eluted with the ATP peak (Fig. 2*D*). Gel analysis showed that G$_t\beta\gamma$ had been lost from the phosducin peak and had reappeared in the 11.8 min peak (Fig. 3*F*, *lanes j* and *l*). Thus, in this column matrix, phosphorylation inhibits phosducin's ability to bind G$_t\beta\gamma$.

The data from the size exclusion column gives a qualitative comparison of dephospho- and phosphophosducin binding to G$_t\beta\gamma$. In order to further quantify the differences in binding affinity between the two forms of phosducin, a second experimental approach was employed. This method took advantage of the fact that, unlike free G$_t\beta\gamma$, the phosducin-G$_t\beta\gamma$ complex does not bind to ROS membranes (15). G$_t\beta\gamma$ was iodinated with [^{125}I]Bolton-Hunter reagent in order to accurately determine amounts of G$_t\beta\gamma$ on the membrane and in the supernatant. ^{125}I-G$_t\beta\gamma$ was incubated with increasing concentrations of phosducin in the presence of unilluminated urea-stripped ROS membranes at a 10-fold excess of Rho over ^{125}I-G$_t\beta\gamma$. After incubation, the ROS membranes were pelleted, and the amount of ^{125}I-G$_t\beta\gamma$ in the pellet was quantified. In the absence of phosducin, 3.2 pmol, or 46% of the total ^{125}I-G$_t\beta\gamma$ was found in the pellet (Fig. 3). The addition of phosducin inhibited ^{125}I-G$_t\beta\gamma$ binding to the membrane almost completely. Fitting the data to a simple model for 1:1 stoichiometric binding of ^{125}I-G$_t\beta\gamma$ to phosducin indicated that, at saturation, 0.48 pmol of ^{125}I-G$_t\beta\gamma$ were in the membrane pellet and that the K_d for phosducin binding to ^{125}I-G$_t\beta\gamma$ was 0.11 μM. Inhibition of ^{125}I-G$_t\beta\gamma$ binding to the membrane was not caused by phosducin competing for the same membrane binding sites as ^{125}I-G$_t\beta\gamma$ because phosducin did not bind to the membrane. SDS-PAGE analysis showed < 5% of the total phosducin was found in the membrane pellet (Fig. 4*B*, *lane b*). Thus, the most compelling explanation of the inhibition of ^{125}I-G$_t\beta\gamma$ binding to the membrane is that it reflects conformational changes resulting directly from phosducin-^{125}I-G$_t\beta\gamma$ interaction.

When phosducin was phosphorylated by cAMP-dependent protein kinase, only small decreases in the binding of ^{125}I-G$_t\beta\gamma$ were observed (K_d = 0.18 μM), and the saturation point for inhibition of ^{125}I-G$_t\beta\gamma$ binding to the membrane was similar (0.58 pmol in the pellet) to that of dephosphophosducin. Thus, phosphophosducin bound ^{125}I-G$_t\beta\gamma$ nearly as well as did dephosphophosducin. Iodination did not appear to change the

interaction of $G_t\beta\gamma$ with phosducin, because similar results were obtained with unlabeled $G_t\beta\gamma$ (measured by SDS-PAGE analysis of membrane bound and supernatant fractions). Moreover, unlabeled $G_t\beta\gamma$ competed equally well with ^{125}I-$G_t\beta\gamma$ for phosducin binding in membrane binding experiments (data not shown).

Effect of $G_t\alpha$ on Phosducin Binding to $G_t\beta\gamma$—The small decrease in binding affinity of phosphophosducin for $G_t\beta\gamma$ could not explain the striking difference in migration of $G_t\beta\gamma$ with the two forms of phosducin on the size exclusion column, nor could it explain the large difference in the ability of dephospho- and phosphophosducin to inhibit ^{125}I-$G_t\alpha/G\beta\gamma$ binding to Rho*. Thus, it appeared that phosphorylation of phosducin was regulating phosducin's inhibition of G_t activation by Rho* at a site different from phosducin-$G_t\beta\gamma$ binding. Therefore, the effect of $G_t\alpha$ on the binding of dephospho- and phosphophosducin to $G_t\beta\gamma$ was measured. Increasing amounts of $G_t\alpha$ were added to ^{125}I-$G_t\beta\gamma$ and phosducin in the membrane binding experimental format described above (Fig. 4*A*). In the absence of phosducin, $G_t\alpha$ caused an increase of ^{125}I-$G_t\beta\gamma$ binding to unilluminated urea-stripped ROS membranes, as evidenced by an increase in ^{125}I-$G_t\beta\gamma$ in the membrane pellet from 2.8 to 4.0 pmol. This result suggests that $G_t\alpha\beta\gamma$ binds ROS membranes with higher affinity than $G_t\beta\gamma$ alone. In the presence of 2 μM phosphophosducin, the binding of ^{125}I-$G_t\beta\gamma$ to the membrane was inhibited as expected. Only 0.7 pmol of the ^{125}I-$G_t\beta\gamma$ remained in the membrane pellet. When $G_t\alpha$ was added, a large increase in ^{125}I-$G_t\beta\gamma$ binding to the membrane was observed. ^{125}I-$G_t\beta\gamma$ in the membrane pellet increased from 0.7 to 4.0 pmol, reaching the level found in the absence of phosducin. These data indicate that $G_t\alpha$ does compete with phosphophosducin for binding to $G_t\beta\gamma$, and that at ~5-fold excess $G_t\alpha$ the phosphophosducin is completely displaced by $G_t\alpha$.

$G_t\alpha$ competition with dephosphophosducin for ^{125}I-$G_t\beta\gamma$ binding was more complex. In the presence of 2 μM dephosphophosducin, $G_t\alpha$ caused a more modest increase in ^{125}I-$G_t\beta\gamma$ binding to the membrane (from 0.5 to 2.0 pmol). The initial increase in bound ^{125}I-$G_t\beta\gamma$ paralleled that of phosphophosducin, but then it leveled off at 2.0 pmol ^{125}I-$G_t\beta\gamma$ in the pellet, compared with 4.0 pmol in the absence of phosducin or with phosphophosducin. Thus, this increase in bound ^{125}I-$G_t\beta\gamma$ does not appear to result from direct competition of $G_t\alpha$ with dephosphophosducin for binding to ^{125}I-$G_t\beta\gamma$. The increase also does not appear to result from increased binding of free ^{125}I-$G_t\beta\gamma$ in the presence of $G_t\alpha$, as was observed in the absence of phosducin. That effect exhibited a $K_{½}$ of 0.4 μM which is equal to the ^{125}I-$G_t\beta\gamma$ concentration, whereas the increased ^{125}I-$G_t\beta\gamma$ binding in the presence of dephosphophosducin had a $K_{½}$ of 1.8 μM. Moreover, very little free ^{125}I-$G_t\beta\gamma$ exists at 2 μM phosducin and could not account for the observed 1.5 pmol increase in membrane-bound ^{125}I-$G_t\beta\gamma$.

This observation might suggest the formation of phosducin-$G_t\alpha\beta\gamma$ complexes that bind $G_t\alpha$ with lower affinity than does $G_t\alpha\beta\gamma$, and which associate with ROS membranes with higher affinity than does phosducin-$G_t\beta\gamma$. While phosducin-$G_t\alpha\beta\gamma$ complexes have been proposed (12), no compelling evidence for such a complex has been presented. SDS-PAGE analysis of phosducin binding to the membrane pellet in the presence of excess $G_t\beta\gamma$ showed no bound phosducin in the absence or presence of $G_t\alpha$, indicating that $G_t\alpha$ does not increase phosducin binding to the membrane (Fig. 4*B*). In contrast, significant amounts of both $G_t\alpha$ and $G_t\beta\gamma$ were found in the membrane pellet, and $G_t\alpha$ caused an increase in $G_t\beta\gamma$ binding to the membrane as expected. Thus, the observed increase in ^{125}I-$G_t\beta\gamma$ binding in the presence of dephosphophosducin and $G_t\alpha$ cannot be explained by the formation of a phosducin-$G_t\alpha\beta\gamma$ complex.

An alternative explanation for the data is that the different isoforms of phosducin compete with $G_t\alpha$ for $G_t\beta\gamma$ binding with different affinities. One isoform might be more readily displaced from $G_t\beta\gamma$ by $G_t\alpha$ in a manner analogous to the displacement of phosphophosducin, whereas other isoforms might not be displaced by $G_t\alpha$. This could account for the partial increase in ^{125}I-$G_t\beta\gamma$ in the membrane pellet when $G_t\alpha$ is added in the presence of dephosphophosducin.

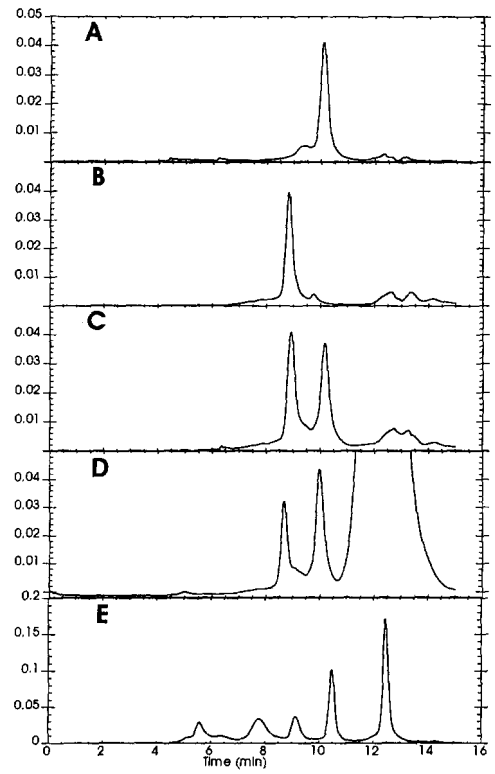

FIG. 5. **HPLC analysis of interactions of phosducin and $G_t\alpha$.** HPLC size exclusion chromatography and gel analysis of peak fractions was performed as described under "Experimental Procedures." Elution profiles from the size exclusion column are as follows: *A*, 0.6 nmol of $G_t\alpha$; *B*, 0.6 nmol of phosducin; *C*, 0.6 nmol of phosducin and 0.6 nmol of $G_t\alpha$; *D*, 0.6 nmol of phosphophosducin, 0.6 nmol of $G_t\alpha$, 200 units of cAMP-dependent protein kinase, 12 nmol of ATP; *E*, molecular mass standards (see Fig. 2*E*) with the following retention times; 5.6 min, bovine thyroglobulin; 7.8 min, bovine γ globulin; 9.2 min, chicken ovalbumin; 10.5 min, horse myoglobin; 12.5 min, vitamin B-12. In *panel C*, phosducin and $G_t\alpha$ were incubated for 5 min at 23 °C prior to sample injection. In *panel D*, phosphophosducin was prepared (see "Experimental Procedures"), and then $G_t\alpha$ was added and incubated for 5 min at 23 °C prior to sample injection.

Regardless of the reason behind partial competition between dephosphophosducin and $G_t\alpha$, the principal conclusion drawn from these experiments is that the $G_t\alpha{\cdot}G_t\beta\gamma$ interaction is the locus at which phosphorylation controls phosducin inhibition of G_t activation. Phosphophosducin is displaced from $G_t\beta\gamma$ by $G_t\alpha$ and, therefore, does not inhibit G_t activation by Rho*. In contrast, dephosphophosducin is not readily displaced from $G_t\beta\gamma$ by $G_t\alpha$ and, therefore, blocks G_t activation by Rho* through sequestration of $G_t\beta\gamma$.

Assessment of $G_t\alpha$ Binding to Phosducin—Phosducin has been shown to inhibit the GTPase activity of $G_o\alpha$ in the absence of $G\beta\gamma$ (12), suggesting that phosducin can bind $G_o\alpha$ alone. Size exclusion chromatography (Bio-Silect SEC250) was used to assess phosducin binding to $G_t\alpha$ in the absence of $G_t\beta\gamma$ (Fig. 5). $G_t\alpha$ eluted at 10.0 min in the absence of phosducin (Fig. 5*A*) and phosducin eluted at 8.8 min in the absence of $G_t\alpha$ (Fig. 5*B*). When the two were incubated together prior to injection into the column, no change in elution times or peak areas for either phosducin or $G_t\alpha$ was observed (Fig. 5*C*). This is in sharp con-

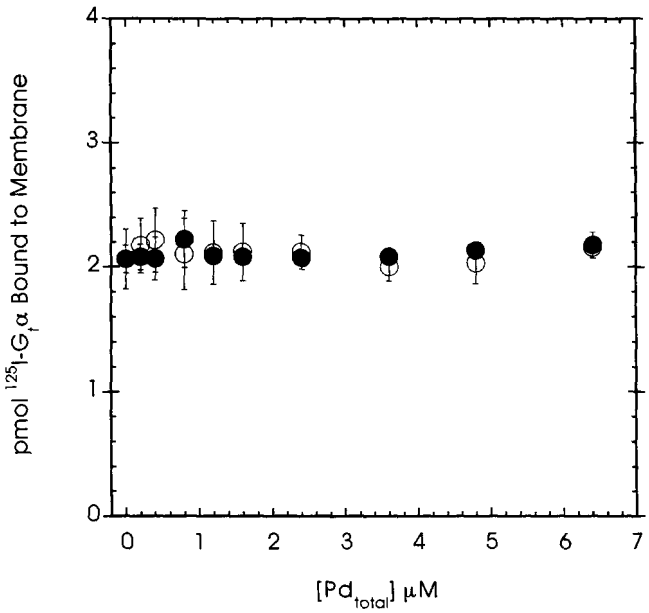

FIG. 6. **Effect of phosducin on ^{125}I-G$_t\alpha$ binding to ROS membranes.** The binding of ^{125}I-G$_t\alpha$ (0.35 μM) to urea-stripped ROS membranes (40 μM Rho) was measured in the presence of increasing concentrations of dephospho- (○) or phosphophosducin (●) as indicated (see "Experimental Procedures"). The total ^{125}I-G$_t\alpha$ in the sample was 7.0 pmol. *Error bars* represent the standard error of the data from two separate experiments and are representative of data from two different phosducin preparations.

trast to the elution profile of phosducin plus G$_t\beta\gamma$, in which all the G$_t\beta\gamma$ co-eluted with phosducin (Fig. 2*C*). In addition, phosphorylation of phosducin with cAMP-dependent protein kinase and ATP did not change the phosducin plus G$_t\alpha$ elution profile (Fig. 5*D*).

The possibility of a phosducin-G$_t\alpha$ interaction was further probed by examining the effect of phosducin on the intrinsic affinity of G$_t\alpha$ for ROS membranes in the absence of G$_t\beta\gamma$. Fig. 6 shows data for ^{125}I-G$_t\alpha$ binding to ROS membranes in the presence of increasing phosducin concentrations. Since urea-stripped ROS membranes contain residual amounts of endogenous G$_t\beta\gamma$, the membranes were heat denatured prior to measuring ^{125}I-G$_t\alpha$ binding.[2] Phosducin had no effect on ^{125}I-G$_t\alpha$ binding to the heat denatured membranes. Despite the addition of up to ~20-fold excess of phosducin, no change in the amount of ^{125}I-G$_t\alpha$ in the membrane pellet was observed. Moreover, phosphorylation of phosducin did not modify the binding. This result should be contrasted with the large decrease in membrane bound ^{125}I-G$_t\beta\gamma$ (from 3.2 to 0.5 pmol) upon the addition of phosducin. Thus, the data from Figs. 5 and 6 give no evidence for a direct interaction between phosducin and G$_t\alpha$. The reason for the apparent discrepancy between these data and the G$_o\alpha$ GTPase data mentioned above (12) is not obvious. However, it is possible that G$_t\alpha$ and G$_o\alpha$ differ in their ability to bind phosducin so that phosducin interacts differently with different G-proteins.

[2] Heat denaturation decreased the amount of ^{125}I-G$_t\alpha$ in the membrane pellet from 4.2 ± 0.3 pmol (n = 3) to 2.0 ± 0.2 pmol (n = 4) under these conditions. Adding back exogenous G$_t\beta\gamma$ restored ^{125}I-G$_t\alpha$ binding to the membrane, with a $K_{1/2}$ of 0.1 μM, and the effect saturated at 5.6 pmol of the ^{125}I-G$_t\alpha$ in the pellet. Heat denaturation of the membranes had little effect on G$_t\beta\gamma$ binding. 3.1 ± 0.05 pmol (n = 4) and 3.6 ± 0.06 pmol (n = 2) of the ^{125}I-G$_t\beta\gamma$ were in the membrane pellet in unheated and heat denatured membranes, respectively. Thus, it appears that heating urea-stripped ROS membranes in this manner denatures the ~4% of the endogenous G$_t\beta\gamma$ that remains after urea stripping. These results also suggest that G$_t\beta\gamma$ does not bind to proteins in the ROS membrane, which would be denatured by heating. Moreover, endogenous G$_t\beta\gamma$ does not significantly affect the binding of exogenous ^{125}I-G$_t\beta\gamma$ to the membranes under the condition of excess G$_t\beta\gamma$ binding sites used in these experiments.

DISCUSSION

Phosducin inhibits G$_t$ activation by blocking its binding to Rho*, apparently as a result of G$_t\beta\gamma$ sequestration in a phosducin-G$_t\beta\gamma$ complex. A single G$_t\beta\gamma$ can act catalytically to activate many G$_t\alpha$s, because once the Rho*·G$_t\alpha\beta\gamma$ complex dissociates upon GTP binding, the G$_t\beta\gamma$ is freed to interact with other G$_t\alpha$s (23). However, the rate of G$_t\alpha$ activation by Rho* depends on the concentration of free G$_t\beta\gamma$, with maximal activation occurring at a 1:1 ratio of G$_t\alpha$ to G$_t\beta\gamma$ (24). Therefore, sequestering free G$_t\beta\gamma$ in a complex with phosducin would decrease the rate of G$_t\alpha$ activation by Rho* without totally abolishing it. This type of down-regulation is precisely what is observed in light-adapted retinal rods (reviewed in Ref. 25). The phosducin concentration in retinal rods is estimated to be equal to that of G$_t$ (15), so there is sufficient phosducin present to substantially decrease G$_t$ activation.

Although it has been suggested that phosphorylation of phosducin may regulate its function in retinal rods (16), the consequences of phosphorylation had been unclear. This may reflect the fact that the binding affinities of phospho- and dephosphophosducin for purified G$_t\beta\gamma$ are similar. The data presented here show that phosphorylation of phosducin regulates its ability to block G$_t\alpha$ interaction with G$_t\beta\gamma$. Phosphophosducin binds G$_t\beta\gamma$ with similar affinity to dephosphophosducin but in a conformation that allows G$_t\alpha$ to interact with G$_t\beta\gamma$ and displace phosphophosducin. Once formed, the G$_t\alpha\beta\gamma$ can be effectively activated by Rho*. In the unphosphorylated state, phosducin interacts with G$_t\beta\gamma$ in a conformation that excludes G$_t\alpha$ from accessing its binding site on G$_t\beta\gamma$, and, therefore, G$_t\alpha$ cannot displace dephosphophosducin.

Evidence for two different conformations of phosducin-G$_t\beta\gamma$, depending on the phosphorylation state of phosducin stems from their contrasting behavior on the size exclusion column. G$_t\beta\gamma$ in the absence of phosducin interacted with the column matrix; but in the presence of phosducin, G$_t\beta\gamma$ was bound in a conformation that did not allow it to interact with the column matrix, and the two proteins migrated in a phosducin-G$_t\beta\gamma$ complex. When phosducin was phosphorylated, despite having a similar affinity for G$_t\beta\gamma$, phosphophosducin and G$_t\beta\gamma$ were separated on the column. The best explanation for this result is that phosphophosducin bound G$_t\beta\gamma$ in a different conformation, which allowed G$_t\beta\gamma$ to interact with the column matrix. Such an interaction would effectively pull G$_t\beta\gamma$ from phosphophosducin during the column run. It is possible that G$_t\alpha$ acts in a manner similar to the column matrix in distinguishing between dephospho- and phosphophosducin-G$_t\beta\gamma$ complexes.

From these findings, a model depicting phosducin as a phosphorylation-dependent regulator of light-activated G-protein mediated-signaling in retinal rods can now be proposed (Fig. 7). In unilluminated rods, cyclic nucleotide levels are elevated (26–28), cAMP-dependent protein kinase is active (29, 30), and phosducin remains phosphorylated (17). Under these conditions, phosphophosducin cannot compete with G$_t\alpha$ for G$_t\beta\gamma$ binding. Therefore, G$_t\alpha$ and G$_t\beta\gamma$ are maximally associated in their active heterotrimeric state. After illumination, cyclic nucleotide levels fall (28, 31, 32), cAMP-dependent protein kinase is inactivated, and phosducin is dephosphorylated by unopposed rod phosphatases (17, 33). Dephosphophosducin binds G$_t\beta\gamma$ and prevents G$_t\alpha$ from associating with G$_t\beta\gamma$, thereby constraining Rho* activation of G$_t\alpha$ and slowing the rate of cGMP-phosphodiesterase activation by G$_t\alpha$·GTP. Thus, phosducin could act in concert with increased guanylyl cyclase (34) and rhodopsin kinase (35) activities (following light-induced

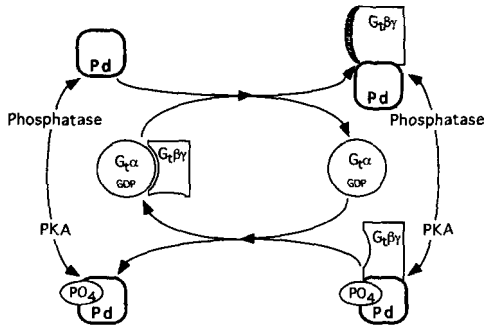

FIG. 7. **Model of phosducin regulation of G-protein dynamics.** The sketch depicts the proposed competition between $G_t\alpha$ and phosducin for binding to $G_t\beta\gamma$. When phosducin is not phosphorylated, it binds $G_t\beta\gamma$ in a conformation that masks the $G_t\alpha$ binding site, effectively blocking $G_t\alpha$ binding to $G_t\beta\gamma$. When phosducin is phosphorylated, it still binds $G_t\beta\gamma$, but it does not mask the $G_t\alpha$ binding site. Thus, in the presence of $G_t\alpha$, phosphophosducin is displaced, and $G_t\beta\gamma$ reassociates with $G_t\alpha$. See text for further details of the model. *Pd*, phosducin; *phosphatase*, phosphatase 2A (of which phosducin has been proposed to be a substrate (33)).

decreases in $[Ca^{2+}]$) to facilitate rod recovery from the light response. In addition, phosducin could also function to down-regulate subsequent light responses until that point in time when cyclic nucleotide levels have been fully restored and phosducin has been rephosphorylated. Once phosphorylated, the phosphophosducin-$G_t\beta\gamma$ complex becomes unstable in the presence of $G_t\alpha$. $G_t\alpha$ binds to $G_t\beta\gamma$ and displaces phosphophosducin. Thus, the system is returned to its dark-adapted state and $G_t\alpha\beta\gamma$ is able to interact efficiently with a Rho* produced by the next photon absorption.

Growing insight into the complexity and number of $G\beta\gamma$ functions has provided a compelling context within which to study the ubiquitous G-protein regulator phosducin. Phosphorylation state-dependent inhibition of $G\alpha$ and $G\beta\gamma$ interactions may be a general mechanism of action of phosducin for all G-proteins. Phosducin might also block activation of enzymes that are directly activated by $G\beta\gamma$, such as β-adrenergic receptor kinase (5), type II adenylyl cyclase (6, 7), and phospholipase C-β (8, 9). Moreover, the effects of phosducin on its target G-proteins are in turn subject to feedback regulation by the impact of the activated G-proteins on adenylyl cyclase and phosphodiesterase. These enzymes determine the cellular concentrations of cyclic nucleotides, the cAMP-dependent protein kinase activity, and, thus, the phosphorylation state of phosducin.

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