Phosducin has recently been identified as a cytosolic protein that interacts with the βγ-subunits of G proteins and thereby may regulate transmembrane signaling. It is expressed predominantly in the retina but also in many other tissues, which raises the question of its potential specificity for retinal versus nonretinal βγ-subunits. We have therefore expressed and purified different combinations of β- and γ-subunits from SF9 cells and also have purified transducin-βγ from bovine retina and a mixture of βγ complexes from bovine brain. Their interactions with phosducin were determined in a variety of assays for βγ function: support of ADP-ribosylation of αo by pertussis toxin, enhancement of the GTPase activity of αo, and enhancement of rhodopsin phosphorylation by the β-adrenergic receptor kinase 1 (βARK1). There were only moderate differences in the effects of the various βγ complexes alone on αo, but there were marked differences in their ability to support βARK1 catalyzed rhodopsin phosphorylation. Phosducin inhibited all βγ-mediated effects and showed little specificity toward specific defined βγ complexes with the exception of transducin-βγ (1), which was inhibited more efficiently than the other βγ combinations. In a direct binding assay, there was no apparent selectivity of phosducin for any βγ combination tested. Thus, in contrast to βARK1, phosducin does not appear to discriminate strongly between different G protein β- and γ-subunits.

Guanine nucleotide-binding proteins (G proteins) are transducers between heptahelical receptors and various effectors. Traditionally, G proteins have been classified according to their signal transducing abilities between heptahelical receptors and various effectors. Gi, Gs, Go, Gt, stimulatory, inhibitory, "other," and transducin GTP-binding proteins; PH domain, pleckstrin-homology domain.
the primary interaction partners for phosducin, the aim of the present investigation was to examine the specificity of interactions between defined G protein βγ combinations and phosducin.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of G Protein βγ-Subunits—The generation of recombinant baculoviruses for the G protein subunits β1, β2, γ2, and γ, has been described earlier (22). For large scale preparations of recombinant βγ-subunits, 200 ml of suspension cultures of Sf9 cells (2 × 10⁵ cells/ml) were co-infected with the recombinant baculoviruses with a multiplicity of infection of 5 or 10 for the β- and the γ-subunits, respectively. The expression of the β- and γ-subunits was monitored with Western blots using antibodies recognizing conserved epitopes of the respective subunits. The blots were developed with peroxidase-coupled goat-anti-rabbit IgG and visualized using ECL reagents (Amersham Corp.). The cells were harvested 72 h after infection, and the βγ complexes were purified from the membranes by solubilization with cholate and chromatography over C7 heptylamine-Sepharose and DEAE-Sepharose columns as described previously (22). The purified βγ complexes were quantitated according to Bradford (24) and also from Coomassie-stained SDS-polyacrylamide gels. Purified βγ complexes were concentrated to about 35 pmol/µl and were stored at -80 °C with 5% glycerol.

G protein βγ-subunits and α from bovine brain and transducin-βγ-subunits from bovine retina were purified according to established procedures (25, 26). In order to reduce the contamination of α with βγ-subunits to a minimum, the usual purification process was extended by a further chromatography step on a C7 heptylamine-Sepharose column.

Phosducin was expressed in Escherichia coli and purified following the procedure of Bauer et al. (8).

ADP-ribosylation of α—The activity of the recombinant βγ-subunits was measured by their ability to support the ADP-ribosylation of α by pertussis toxin. The assay was performed as described by Hekman et al. (27) with minor modifications. 2 pmol of α (40 nm) was used as the substrate in an assay volume of 50 µl containing 600 ng of pertussis toxin (Sigma) and increasing concentrations (2–60 nm) of βγ-subunits. For inhibition of the ADP-ribosylation, 0.8 pmol of α (16 nm) was incubated with 0.8 pmol of βγ-subunits (16 nm), and phosducin was present at concentrations of 8–240 nm (0.4–12 pmol).

GTPase Activity of α—The GTPase activity of α was determined as described elsewhere (6, 28). Reaction mixtures (100 µl) contained 0.1 pmol of α and 0.1–2.4 pmol of βγ complexes (1–24 nm). The effects of phosducin were examined at a constant concentration of βγ-subunits (24 nm) and 24–720 nm phosducin.

Phosphorylation of Rhodopsin—Urea-treated rod outer segments containing >95% rhodopsin (29) were phosphorylated by purified recombinant βγARK1 essentially as described earlier (30). The reaction mixtures (60 µl) contained 50 µM [γ-32P]ATP (Amersham Corp.), 10 pmol (167 nm) rhodopsin, 0.3 pmol (5 nm) of βγRK, 3 pmol (50 nm) of βγ-subunits, and 1.5–60 pmol (25–1000 nm) of phosducin. The incubation was carried out at 30 °C for 8 min under bright white light. The reaction was stopped, and the samples were analyzed by electrophoresis, autoradiography, and Cerenkov counting of the rhodopsin bands.

Phosducin Binding Assay—Wells of microtiter plates were coated with 300 ng of βγ-subunits for at least 4 h at 4 °C in 100 µl of 20 mM Hepes, 20 mM NaCl, 0.1 mM EDTA, pH 7.6, and 0.05% cholate (-incubation buffer). The wells were then washed several times with the same ice-cold buffer supplemented with 0.05% Tween 20 (-wash buffer). After blocking with 3% bovine serum albumin in wash buffer, 0.1–20 µg of phosducin (30 nm to 6 µM) were incubated in the wells at 4 °C for 2 h in 100 µl of incubation buffer plus 5 mM MgCl₂. The wells were then washed and blocked as above. Bound phosducin was determined by addition of affinity-purified rabbit anti-phosducin antibodies for 1 h at room temperature. After incubation with peroxidase-coupled goat-anti-rabbit IgG a color reaction was evoked with o-phenylenediamine dihydrochloride (Sigma) and stopped with 50 µl of 3 M sulfuric acid, and absorption was measured at 490 nm.

**RESULTS**

In order to investigate effects of βγ-subunits of defined composition, the proteins were produced in Sf9 cells by co-infection with recombinant baculoviruses directing the expression of defined β- and γ-subunits. The resultant βγ complexes were then purified to >95% purity by chromatography on C7 heptylamine-Sepharose and DEAE-Sepharose columns as described previously (22). The βγ complexes were used in this study were β₂γ₂, β₂γ₁, and β₃γ₂. Mock preparations of cells infected with wild-type baculoviruses were prepared in the same manner for control purposes. Transducin-βγ (β₁γ₁) and a bovine brain βγ preparation containing multiple β- as well as γ-subunits were prepared by established procedures to serve as controls.

All βγ preparations were able to support pertussis toxin-catalyzed ADP-ribosylation of α. (Fig. 1A). There were no major differences between the different recombinant βγ complexes. The two native preparations, transducin-βγ and the mixed preparation of βγ-subunits from bovine brain, were more effective at lower concentrations than the recombinant preparations; at higher concentrations this difference disappeared for transducin-βγ and became small for the bovine brain βγ preparation.

Phosducin inhibited these enhancing effects of all βγ complexes on pertussis toxin-catalyzed ADP-ribosylation of α. (Fig. 1B) shows the phosducin-mediated inhibition of ADP-ribosyla-
Interactions of Phosducin with βγ-Subunits

**Fig. 2. Stimulation of the GTPase activity of αo by βγ-subunits (panel A) and its inhibition by phosducin (panel B).** A, the GTPase of αo (1 nM) was stimulated in the presence of high Mg^2+ (10 mM) with increasing concentrations of different βγ-dimers (1 to 25 nM). Nonlinear curve fitting to the Hill equation gave the following E_{max} and IC_{50} values: ββγ, 614 ± 44%; βγγ, 347 ± 107%; β2γγ, 24.6 ± 12.8 nM; βγγ, 338 ± 18%; βγγ, 15.8 ± 1.7 nM; βγγ, 331 ± 27%; βγγ, 24 ± 3.3 nM; βγγ, 751 ± 163%; β3γ, 47 ± 14 nM; βγγ, 235 ± 52%; βγγ, 16.5 ± 6.9 nM. Data are means ± S.E. of five experiments. B, the stimulatory effect of βγ complexes on 1 nM αo (set to 100%) was antagonized with various concentrations of phosducin (24 to 720 nM). L_{max} and IC_{50} values were calculated using nonlinear curve fitting according to the Hill equation: βγγ, 65 ± 3.6%; βγγ, 31 ± 7.7 nM; βγγ, 60 ± 4.2%; βγγ, 46 ± 13.1 nM; βγγ, 58 ± 8.8%; βγγ, 26 ± 19 nM; βγγ, 71 ± 1.6%; βγγ, 48 ± 4.4 nM; βγγ, 47 ± 4.8%; βγγ, 165 ± 49 nM; βγγ, 87 ± 11.6%; βγγ, 44 ± 2.4 nM. Data are means ± S.E. of six experiments.

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G protein-mediated signaling systems comprise a series of proteins, including the receptors, the G protein subunits, the effectors and various partially cytosolic proteins. The steadily increasing number of isoforms for all of these proteins that are being discovered raises the question of protein-protein specificity. A fairly consistent pattern is the specificity of the proteins that are involved in phototransduction: rhodopsin, G\(_\alpha\), cGMP-phosphodiesterase, rhodopsin kinase, and arrestin are all expressed only in the retina (and in the developmentally related pineal gland). Furthermore, in in vitro assays they show marked specificity toward each other. For example, rhodopsin kinase is much better in phosphorylating rhodopsin than are \(\beta\)-adrenergic receptors, whereas the reverse is true for \(\beta\)-adrenergic receptor kinase (32). Similarly, arrestin binds much better to rhodopsin than to \(\beta\)-adrenergic receptors, while \(\beta\)-arrestin prefers \(\beta\)-adrenergic receptors (28). At the level of the signaling chain itself (receptor/G protein/effectors) the issue of specificity is less clear-cut. While a series of experiments with antisense oligonucleotides against individual G protein \(\alpha\), \(\beta\)- and \(\gamma\)-subunits have suggested a remarkable specificity for certain receptor/ion channel coupling (33–36), reconstitution experiments have shown only moderate specificity of receptor/G protein coupling and various degrees of specificity for regulation of effectors by G protein subunits (19, 20, 22, 37, 38).

Phosducin differs from the other proteins of such signaling chains in its expression pattern: while it is very abundant in the retina and in the pineal gland, it is also expressed in many other tissues (8). Since phosducin interacts primarily with the retinal tissues would makes sense only if it exhibited little or no selectivity toward specific \(\beta\)-subunits.

This question was addressed in the present study by investigating the interaction of phosducin with a number of defined G protein \(\beta\) subcomplexes in several functional assays as well as in a direct binding assay. For this purpose, defined \(\beta\) subcomplexes were produced in Sf9 cells with the help of recombinant baculoviruses. The baculovirus expression system has been used by several groups for producing functional G protein subunits in high quantities (19, 22, 39, 40). For the synthesis of functional \(\beta\)-subunits it is necessary to express both subunits simultaneously. Their functionality can be ascertained by their ability to support the ADP-ribosylation of G protein \(\alpha\) subunits by pertussis toxin. In our hands, all the combinations tested so far were able to enhance the ADP-ribosylation of \(\alpha\) equally well. We interpret this finding as evidence for equal functional activity of the different \(\beta\) preparations. Similar observations have been made by others regarding the ADP-ribosylation of native \(\alpha\) and \(\alpha\), whereas for \(\alpha\), produced in E. coli (which was not myristoylated) transducin-\(\beta\) was less efficient and potent (19, 20, 37).
Interactions of Phosducin with βγ-Subunits

Another functional test for βγ complexes that likewise involves interaction with the α-subunits is their ability to alter the intrinsic GTPase activity of the α-subunit. In the case of αo, βγ-subunits activate the GTPase in the presence of high concentrations of Mg²⁺ whereas in the presence of low concentrations of Mg²⁺ (below 1 mM) they exert an inhibitory effect (31). In our studies, we measured modest differences in the activity of the different βγ dimers on αo, which were about 2-fold for the best (β₁γ₂) versus the worst (transducin-βγ) combination. The pattern of selectivity was somewhat different from the one found in the ADP-ribosylation assays, suggesting that there are different requirements for the βγ-subunits in the two different types of experiments. Investigations by Ueda et al. (20) on the inhibition of α-subunit GTase activity by βγ-subunits have also failed to reveal significant selectivity with the exception of β₁γ₂, which was less potent in all assays.

A third, quite different test for the functionality of the βγ complexes is their ability to serve as membrane anchors for βARK to enhance agonist-dependent receptor phosphorylation (3, 4). In an earlier study we had found differences between defined βγ complexes in their ability to enhance βARK-mediated rhodopsin phosphorylation (22). Similar differences were found here, with β₂γ₂ being most efficient and transducin-βγ least.

Phosducin had inhibitory effects on all of these βγ-mediated effects. Among the different βγ combinations, the stimulatory effects of transducin-βγ both on ADP-ribosylation and GTPase activity were more efficiently antagonized, while we did not detect any real differences between the recombinant dimers consisting of β₁γ₁, β₁γ₂ and γ₂. Phosducin also inhibited the βγ-mediated enhancement of rhodopsin phosphorylation. However, the divergent enhancing potential of the βγ complexes allowed a characterization of this inhibition only in the case of β₁γ₂, β₂γ₂ and βγ.

While stimulation of rhodopsin phosphorylation by β₁γ₂ or β₂γ₂ was equally well inhibited by phosducin, the effect on βγ₂ was less pronounced and appeared to require higher concentrations of phosducin. Similarly, we also observed only a weak inhibition on the βγ₂-mediated stimulation of αo GTase. We speculate that the mixture of βγ-subunits from bovine brain might contain βγ complexes which are only poorly recognized by phosducin.

Whereas the functional assays suggested a preference of phosducin for transducin-βγ, we found no real differences in affinity or maximal binding when we assayed phosducin binding to immobilized βγ-subunits directly. Thus, the apparent selectivity of phosducin for transducin-βγ in the functional assays may be caused by events which occur subsequent to the initial binding step. An alternative explanation is that transducin-βγ has a better solubility than the other βγ complexes and that the binding assay was done with immobilized βγ-subunits while the ADP-ribosylation and GTPase assays were carried out in solution. In this context it is important to note that the affinities measured in the direct binding assay with immobilized βγ-subunits were ~10-fold lower than those determined in the functional assays in solution. Likewise, the affinities of phosducin for β₁γ₂ and β₂γ₂ found in the ADP-ribosylation and GTPase assays were ~3-fold higher than those determined in the βARK assay, where the βγ-subunits were integrated in the rod outer segment membranes. Similar discrepancies have been noted earlier. For example, Heitger et al. (41) reported severalfold higher affinities of βγ complexes for αo in detergent-containing solution than in lipid vesicles.

Since transducin-βγ differs from β₁γ₂ or β₂γ₂ only in its γ-subunit, the small functional specificity of phosducin for transducin-βγ must be due to the presence of γ₁. γ₁ contains a different isoprenylation, farnesylation instead of geranylgeranyl in all other G protein γ-subunits. The functional importance of this farnesyl, compared to other prenyl modifications for the interaction with receptors, has very recently been elucidated by Kisselev et al. (42). Alternatively, the slightly different effects of γ₁ may be due to its rather different amino acid sequence: the amino acid identity of γ₁ with γ₂ or γ₃ is less than 40%, while the sequence identity between γ₂ and γ₃ is about 80% (18).

Overall, however, the interactions of phosducin with the various βγ complexes appear to be quite similar. This relative lack of specificity goes in line with the nonselective expression of phosducin, since it enables an interaction of phosducin with multiple G proteins and thus an interference with multiple signaling pathways. It contrasts with the more pronounced selectivity of βARK for certain βγ complexes were β₂γ₂ was the preferred partner and transducin-βγ ineffective (22) (see also Fig. 3A). This agrees with our hypothesis mentioned in the introduction that the βγ-binding domains of βARK and phosducin are dissimilar. While some homologies between the N terminus of phosducin and the C terminus of βARK have been noted (14, 15), phosducin contains neither a PH domain nor the βγ-binding consensus sequence Glu/Asn-X-Glu/Asp-Arg/Lys proposed by Chen et al. (12). Furthermore, phosducin-like protein (43) with its much shorter N terminus which shows no similarity at all to the C terminus of βARK, also interacts with G protein βγ-subunits (44), supporting the differences in the βγ-binding mechanisms between phosducin and βARK.

While these data suggest that the binding sites on G protein βγ-subunits for βARK and phosducin might be different, they must clearly be overlapping, since phosducin and βARK have been shown to compete for βγ binding (10). Furthermore, the binding sites for phosducin and for βARK appear to overlap also with the binding site for the α-subunits, since both proteins interfere with coupling between the βγ- and the α-subunits. Thus the βγ-subunits appear to possess various distinct but overlapping binding sites for different proteins.

Reconstitution experiments employing defined G protein βγ complexes have largely failed to reveal much specificity. This contrasts with antisense experiments which indicate that G proteins of very specific α-, β-, and γ-composition are required for efficient coupling of certain receptors to ion channels (33-36). This raises the possibility that selectivity may be encoded in the cellular organization rather than in protein-protein interactions. Further studies are required to determine whether similar rules might also govern the interactions of phosducin with G protein βγ-subunits.

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