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A small region in phosducin inhibits G-protein βγ-subunit function

Klaus Blüml[1], Werner Schnepp[1], Stefan Schröder[1], Michael Beyermann[2], Maria Macias[3], Hartmut Oschkinat[2,3] and Martin J.Lohse[1,4]

[1]Institut für Pharmakologie und Toxikologie der Universität Würzburg, Versbacher Straße 9, 97078 Würzburg, [2]Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Straβe 4, 10315 Berlin and [3]European Molecular Biology Laboratory, Meyerhofstraße 1, 69012 Heidelberg, Germany

[4]Corresponding author

K.Blüml and W.Schnepp contributed equally to this work

G-protein βγ-subunits ($G_{\beta\gamma}$) are active transmembrane signalling components. Their function recently has been observed to be regulated by the cytosolic protein phosducin. We show here that a small fragment (amino acids 215–232) contained in the C-terminus of phosducin is sufficient for high-affinity interactions with $G_{\beta\gamma}$. Corresponding peptides not only disrupt $G_{\beta\gamma}$–G_{α} interactions, as defined by $G_{\beta\gamma}$-stimulated GTPase activity of α_o, but also other $G_{\beta\gamma}$-mediated functions. The NMR structure of a peptide encompassing this region shows a loop exposing the side chains of Glu223 and Tyr224, and peptides with a substitution of either of these amino acids show a complete loss of activity towards G_o. Mutation of this Tyr224 to Ala in full-length phosducin reduced the functional activity of phosducin to that of phosducin's isolated N-terminus, indicating the importance of this residue within the short, structurally defined C-terminal segment. This small peptide derived from phosducin may represent a model of a $G_{\beta\gamma}$ inhibitor, and illustrates the potential of small compounds to affect $G_{\beta\gamma}$ functions.

Keywords: adenylyl cyclase/β-adrenergic receptor kinase/G-proteins/phosducin/protein NMR

Introduction

G-proteins act as signal transducers of many membrane-bound, heptahelical receptors. They consist of a GTP-binding α-subunit and a tightly bound complex composed of β- and γ-subunits. When G-proteins are activated by their respective receptors, the α-subunits exchange GDP for GTP, and this causes dissociation of α_{GTP} and the βγ complex, both of which can then regulate the activity of various effector molecules such as adenylyl cyclases or ion channels (reviewed in Birnbaumer, 1992; Hepler and Gilman, 1992; Müller and Lohse, 1995; Neer, 1995). G-protein-mediated signalling is subject to a variety of regulatory controls. Most of these are exerted at the receptor level (reviewed by Hausdorff *et al.*, 1990; Lohse, 1993) but, more recently, evidence has been accumulating that regulation can also occur at the G-protein level.

Several proteins have been described to interact with G-proteins and to alter their activity: the growth cone protein GAP-43 accelerates guanine nucleotide release from G_o, and the RGS (regulators of G-protein signalling) family members have been shown to activate the GTPase activity of the α subunits of G_o or the G_i family (Strittmatter *et al.*, 1991; Berman *et al.*, 1996; Hunt *et al.*, 1996; Watson *et al.*, 1996). A number of other proteins, in particular the β-adrenergic receptor kinases (Pitcher *et al.*, 1992; Koch *et al.*, 1993; Müller *et al.*, 1993), have been shown to bind to G-protein βγ-subunits and this binding has been suggested to be mediated by the C-terminal helix plus adjacent sequences of a conserved structural motif called the pleckstrin homology (PH) domain (Touhara *et al.*, 1994). A consensus motif Gln/Asn-X-X-Glu/Asp-Arg/Lys has been identified in several $G_{\beta\gamma}$-binding effector proteins (β-adrenergic receptor kinase, adenylyl cyclase type 2, phospholipase β-3 and atrial K^+-channels) and has been deemed responsible for mediating this interaction (Chen *et al.*, 1995).

Phosducin is a cytosolic protein which also belongs to the group of $G_{\beta\gamma}$-binding proteins and which appears to act as a regulator of signalling via G-proteins (Bauer *et al.*, 1992; Lee *et al.*, 1992). It occurs in very high concentrations in the retina (Lee *et al.*, 1987) and the developmentally related pineal gland (Reig *et al.*, 1990), but is also expressed in brain and many other tissues at a concentration of ⊤1 μM (Bauer *et al.*, 1992; Danner and Lohse, 1996). These concentrations are sufficient to inhibit G-protein-mediated activation of effectors, such as the receptor-induced stimulation of adenylyl cyclase via G_s (Danner and Lohse, 1996; Schulz *et al.*, 1996). Upon phosphorylation of phosducin by protein kinase A, the inhibitory effects of phosducin on G-proteins are essentially lost (Bauer *et al.*, 1992; Hawes *et al.*, 1994; Yoshida *et al.*, 1994).

Phosducin has a high affinity for $G_{\beta\gamma}$, which is in the range of 20 nM (Bauer *et al.*, 1992; Müller *et al.*, 1996). Two independent studies have suggested that the N-terminal 63 (Xu *et al.*, 1995) or 105 (Hawes *et al.*, 1994) amino acids mediate this interaction. However, the short variant of a phosducin-like protein (PhLP), which lacks the N-terminus but is otherwise ⊤50% identical to phosducin, also interacts with $G_{\beta\gamma}$ with an affinity only 5-fold lower than that of phosducin (Schröder and Lohse, 1996). This suggests the presence of a $G_{\beta\gamma}$-binding region in the large overlapping domain of these two proteins. Here we report the identification and the structure of a small critical region in the C-terminus of phosducin that mediates $G_{\beta\gamma}$ binding.

Results

Mapping of the region in phosducin mediating inhibition of G_o GTPase

Various N-terminally truncated variants of phosducin were generated to map its $G_{\beta\gamma}$-binding site. The first deletion

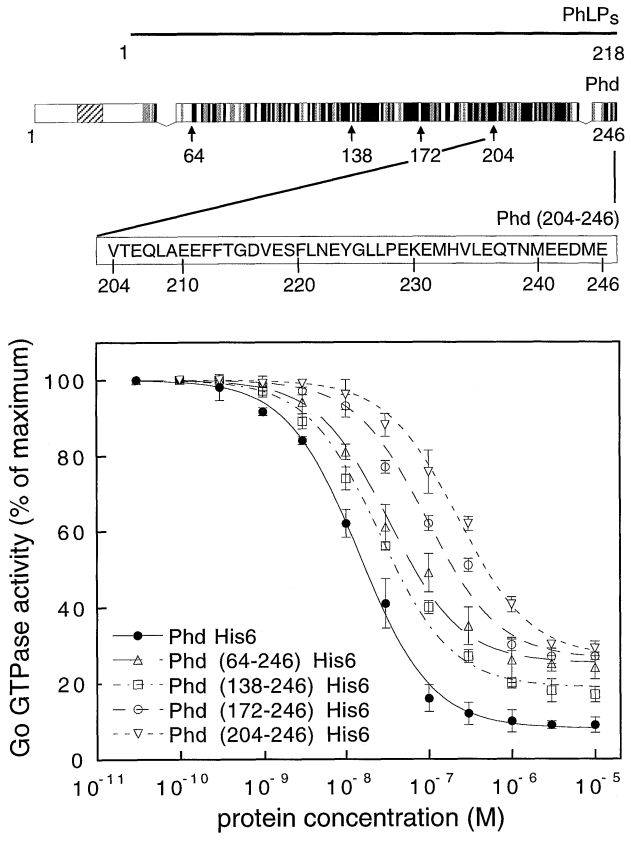

Fig. 1. Inhibition of GTPase activity of G_o by His_6-tagged phosducin and its N-terminally truncated variants. The top bar shows the identities (black), similarities (grey) and gaps (brackets) between phosducin and the long form of phosducin-like protein (PhLP; taken from Schröder and Lohse, 1996). The size of the short form of PhLP is indicated by the line on the top. Analysis with non-linear curve fitting gave the following IC_{50} values: phosducin 15 ± 1 nM, phosducin(64–246) 33 ± 3 nM, phosducin(138–246) 25 ± 2 nM, phosducin(172–246) 95 ± 13 nM; phosducin(204–246) 240 ± 20 nM. Data are means ± SEM of three independent experiments.

(amino acids 1–63) encompasses the N-terminal $G_{\beta\gamma}$-binding site according to Xu *et al.* (1992), and the resultant protein contains the regions of phosducin similar to the short variant of PhLP (Figure 1). Further deletions were done to cover the remaining structure. The full-length and truncated proteins, expressed and purified as C-terminally His_6-tagged proteins, were all capable of inhibiting the GTPase activity of G_o (Figure 1). These inhibitory effects were the same for the Mas-7-stimulated (Higashijima *et al.*, 1990) activity of G_o reconstituted into phospholipid vesicles (Figure 1) and the basal activity of G_o in detergent-containing buffer (see Figure 3). Phosducin itself inhibited the GTPase activity of G_o by ⊤90% with an IC_{50} value of 15 nM. Deletion of the first 63 amino acids caused only a 2-fold loss in affinity, which contrasts with the results from transfection assays obtained by Xu *et al.* (1992). Further N-terminal deletions caused further modest reductions in the potency and efficacy, but even the very C-terminus of phosducin (204–246) had an IC_{50} value of 250 nM. In comparison, the N-terminal fragment phosducin(1–85) had a much higher IC_{50} value of ⊤1 μM in such assays (Figure 8B).

Phosducin itself as well as its small C-terminus displayed direct $G_{\beta\gamma}$ binding: co-incubation of purified $G_{\beta\gamma}$ with His-tagged phosducin in solution allowed specific co-precipitation of $G_{\beta\gamma}$ with a His_6-binding resin (Figure 2). When His-tagged phosducin(204–246) was used instead of phosducin, we also obtained such a signal, even though the intensity of the signal was reduced in accordance with the lower affinity of the short fragment.

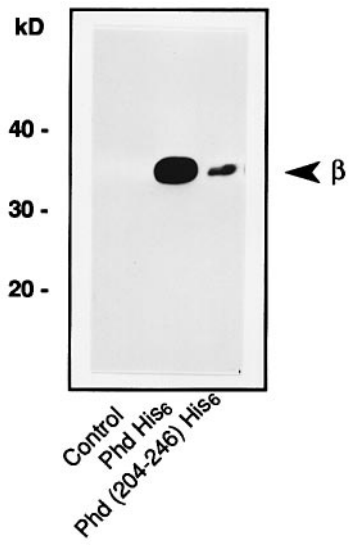

Fig. 2. Direct binding of His_6-tagged phosducin and phosducin(204–246) to $G_{\beta\gamma}$. Purified $G_{\beta\gamma}$ (130 pmol) was incubated alone (left lane) or with 250 pmol of phosducin (middle lane) or phosducin(204–246) on ice, and the His_6-tagged proteins were then bound to Ni-NTA beads and pelleted. After washing, the $G_{\beta\gamma}$ retained in the pellet was detected by SDS–PAGE and Western blotting with antibodies directed against G_β.

Further mapping of the $G_{\beta\gamma}$-binding site was done with synthetic peptides derived from the C-terminus of phosducin (Figure 3A). Two overlapping peptides comprising the active C-terminus as defined above both inhibited the GTPase activity of G_o. The phosducin(204–231) peptide had a slightly higher efficacy and potency than the peptide representing the very C-terminus (amino acids 217–246). These data suggested that the overlapping region of the two peptides contains an essential $G_{\beta\gamma}$-binding site. This conclusion was confirmed by the fact that a peptide containing this overlapping region (amino acids 215–232) was as potent as the larger peptides in inhibiting G_o GTPase activity, with an IC_{50} value of ⊤380 nM. This inhibition of the GTPase activity was strictly dependent on $G_{\beta\gamma}$, because the GTPase activity of isolated α_o was only marginally affected by up to 30 μM phosducin(215–232) (Figure 3B). Considering these peptide data together, phosducin(217–231) appears to contain a high-affinity $G_{\beta\gamma}$-binding region.

Inhibition of other $G_{\beta\gamma}$-mediated effects

$G_{\beta\gamma}$ interacts with multiple proteins other than G_α, and therefore we tested the peptide phosducin(215–232) for its ability to inhibit $G_{\beta\gamma}$ effects on various effector proteins. Since these interactions require cell membranes, we initially determined that the IC_{50} value of this peptide on G_o GTPase in brain membranes was ⊤20 μM (data not shown), indicating that membranes considerably decrease the peptide's potency, probably due to non-specific adsorption of peptides to membrane fragments.

$G_{\beta\gamma}$-stimulated activity of adenylyl cyclase-2 in the

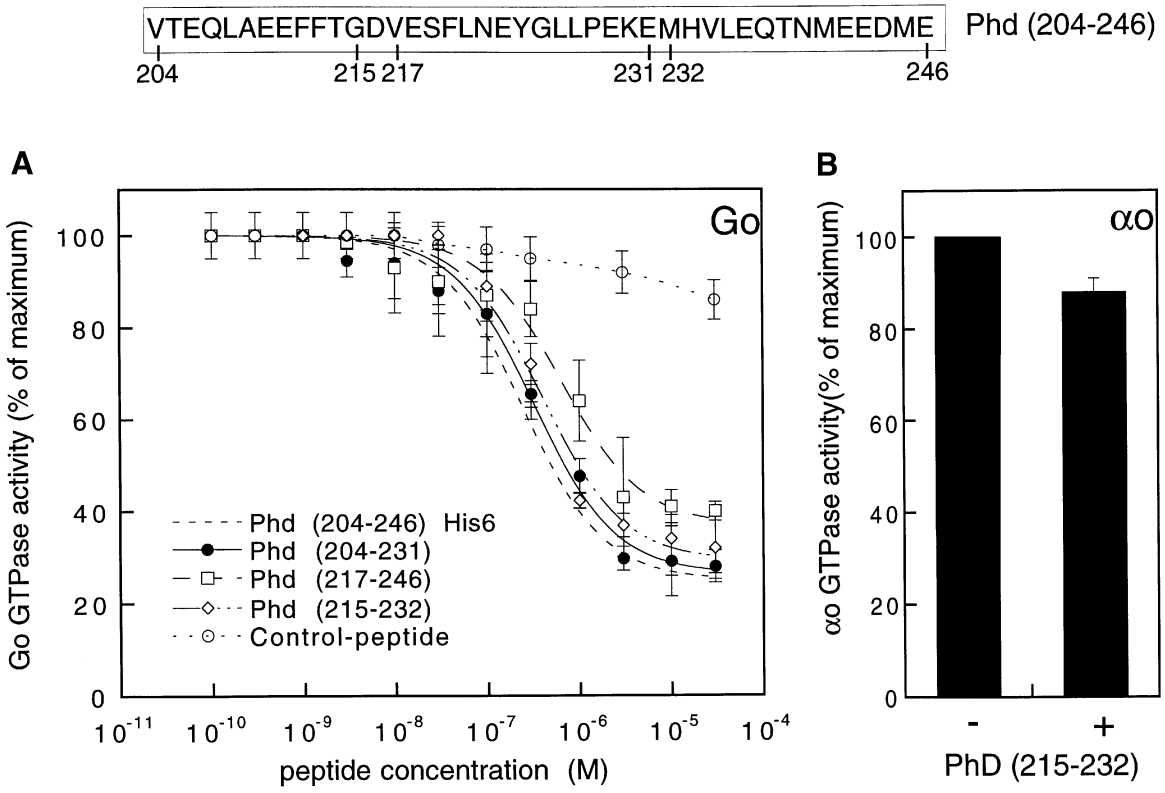

Fig. 3. Inhibition of G_o GTPase activity by synthetic peptides (top) derived from the C-terminus of phosducin. (**A**) The basal GTPase activity of G_o (2 nM) was assayed in solution (in the presence of 0.1% Lubrol and 25 mM $MgSO_4$). The dotted line represents the effects of the His_6-tagged phosducin(204–246). An unrelated peptide (taken from the first intracellular loop of the human β_2-adrenergic receptor; KTAIAKFERLQT-VTNYFITSK) served as a control. IC_{50} values were: phosducin(204–231) 320 ± 45 nM, phosducin(217–246) 630 ± 140 nM, phosducin(215–232) 380 ± 30 nM. Data are means ± SEM of three independent experiments. (**B**) Effect of phosducin(215–232) on the α_o GTPase activity. Isolated α_o (2 nM) in solution was assayed in the absence or presence of 30 μM phosducin(215–232). Data are means ± SEM of three independent experiments.

membranes of Sf9 cells was inhibited by phosducin(215–232) with an IC_{50} value of ⊤100 μM (Figure 4A). In contrast, the activity stimulated with 100 μM forskolin instead of $G_{\beta\gamma}$ was only inhibited by up to 20% (data not shown). Similarly, the peptide at 100 μM reduced the stimulatory effects of $G_{\beta\gamma}$ on the activity of the β-adrenergic receptor kinase and of phospholipase C-β2 (PLC-β2) (Figure 4B and C). These data indicate that multiple $G_{\beta\gamma}$ functions can be affected by the phosducin peptide, even though higher concentrations were required, suggesting that these effects may not all be similar.

NMR structure of the peptide phosducin(204–231)

To elucidate the structural basis for the interaction of this region in phosducin with $G_{\beta\gamma}$, we analysed its structure in NMR experiments with the peptide phosducin(204–231). These studies were done in a physiological buffer at pH 6.5. The two-dimensional ^{1}H-NMR spectra showed a dominantly ordered structure in the region 216–226 as revealed by non-random coil chemical shift values of α- and amide protons, by NOEs of the type $H\alpha_i$-$H\beta_{(i+3)}$ including residues 216–224, by long-range NOEs between F220, L221, N222 and L226 and by a large number of strong NH–NH NOEs (Figures 5 and 6). This ordered state is not populated to 100%, as can be seen from the simultaneous appearance of strong NH–NH and NH–Hα NOEs (Figure 6). This was taken into account in the structure calculations by using more generous upper bounds for this type of NOEs.

The NMR structure of the segment phosducin(215–226) (Figure 7) shows a short α-helical structure comprising residues 216–222, followed by a well-ordered loop region forming a helix cap (amino acids 223 and 224). Hydrophobic interactions between the residues V217, F220, L221, L226 and L227 appear to stabilize its structure.

The importance of this structured region in phosducin for the interaction with and inhibition of $G_{\beta\gamma}$ was established in GTPase assays with a series of variations of the phosducin(215–232) peptide carrying isolated amino acid exchanges (Figure 8A). Replacement of L221 in the centre of the peptide's ordered region by alanine caused only a modest reduction of activity. In contrast, replacement of the tyrosine residue 224 in the turn alone caused a complete loss of activity. Likewise, abolishing the charge in the neighbouring position 223 by a change from glutamate to glutamine caused inactivity of the peptide, indicating the importance of the side chains of this loop. These results suggest that the side chains of E223 and Y224 are essential for the interaction with $G_{\beta\gamma}$.

Further determination of the functional relevance of this structured region was undertaken by introducing the Tyr224→Ala mutation into full-length phosducin. This phosducin mutant showed a considerable loss of functional activity. In fact, its inhibitory activity in the GTPase assays was comparable with that of a fragment containing the

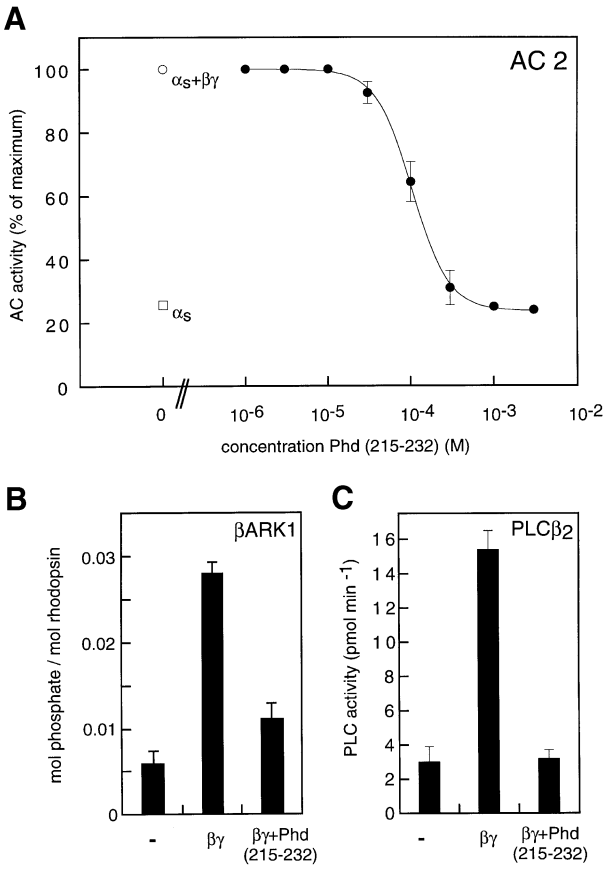

Fig. 4. Inhibition of $G_{\beta\gamma}$-mediated effects by phosducin(215–232). Data are means ± SEM of 3–6 independent determinations. (**A**) Inhibition of $G_{\beta\gamma}$-stimulated adenylyl cyclase-2 activity in the presence of 100 nM $G_{s\alpha}$ and 100 nM $G_{\beta\gamma}$. Five μg (protein) of membranes prepared from Sf9 cells infected with baculovirus for adenylyl cyclase-2 were incubated for 10 min at 30°C with 100 nM GTPγS-activated purified $G_{s\alpha}$ and then for 20 min on ice with 100 nM $G_{\beta\gamma}$, followed by determination of adenylyl cyclase activity. □, Gs_α alone; ○, Gs_α + $G_{\beta\gamma}$; ●, Gs_α + $G_{\beta\gamma}$ + phosducin(215–232). (**B**) Inhibition of rhodopsin phosphorylation by the β-adrenergic receptor kinase in the presence of $G_{\beta\gamma}$. The activity of βARK was determined using rhodopsin in rod outer segments as the substrate as in Schröder and Lohse (1996). The protein concentrations were 1 μM rhodopsin, 2.5 nM βARK-1 and 20 nM $G_{\beta\gamma}$, and incubations lasted for 6 min at 30°C. The concentration of phosducin(215–232) was 100 μM. (**C**) Inhibition of $G_{\beta\gamma}$ (600 nM)-stimulated PLC-β2 activity. PLC-β2 activity was determined with a truncated version of PLC-β2 (PLC-mut) as described by Dietrich *et al.* (1994). [^{3}H]Phosphatidyl 4,5-bisphosphate served as substrate. When present, the concentration of Gβγ was 600 nM, and that of phosducin(215–232) 100 μM. Activity is expressed as pmoles of inositol phosphates formed per minute.

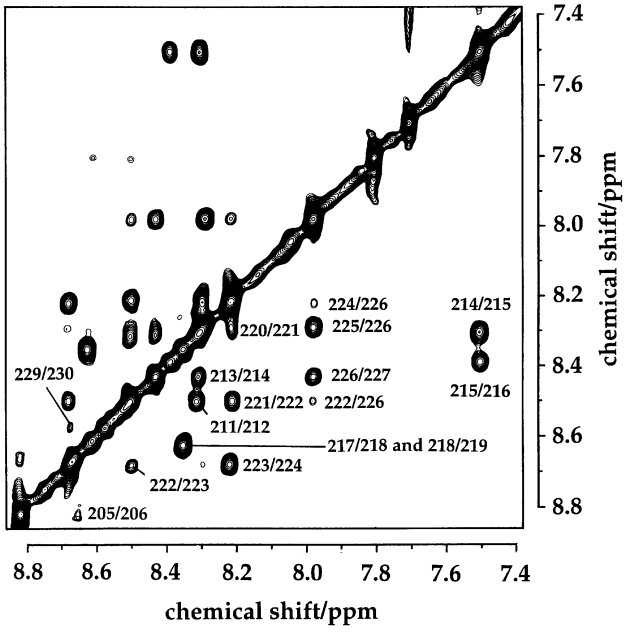

Fig. 5. Amide region of a 500 MHz WATERGATED NOESY spectrum of the peptide phosducin(204–231) in 10 mM phosphate buffer containing 100 mM NaCl and 10% D_2O at pH 6.5 and 277 K. The mixing time used was 250 ms. Numbers denote the corresponding amino acid residues. The strong NH–NH NOEs indicate an ordered state in the middle part of the peptide phosducin(204–231) and were also observed using a mixing time of 40 ms.

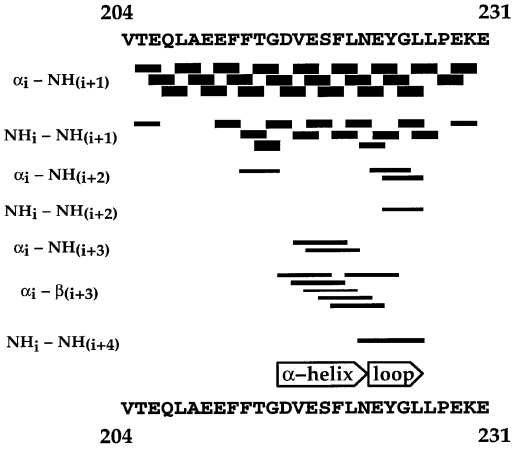

Fig. 6. Peptide sequence of phosducin(204–231) and summary of short and medium range NOEs observed in NOESY spectra. Line thickness is proportional to the signal intensity. NOEs between side chains are not shown. Arrows are used to describe the secondary structure elements.

first 85 N-terminal amino acids of phosducin, suggesting that the entire contribution of the C-terminal phosducin segment was abolished by the Tyr224→Ala mutation (Figure 8B). These data confirm the results obtained with corresponding peptides and underline the importance of this tyrosine residue within the structurally defined region for the interaction with $G_{\beta\gamma}$.

Discussion

The structural and functional basis for the interaction of various proteins with $G_{\beta\gamma}$ recently has attracted much interest. The data presented here indicate that the C-terminus of phosducin contains a minimal $G_{\beta\gamma}$-binding region which conveys submicromolar affinity for $G_{\beta\gamma}$ and disrupts several $G_{\beta\gamma}$-mediated functions. Other studies using GST–phosducin fusion proteins (Hawes *et al.*, 1994; Xu *et al.*, 1995) found no major contribution of regions other than the N-terminus to $G_{\beta\gamma}$ binding. However, experiments comparing phosducin with PhLP (Schröder and Lohse, 1996) as well as the present truncation studies revealed only a 2- to 5-fold increase in the affinity for $G_{\beta\gamma}$ caused by the presence of phosducin's N-terminus, and clearly indicate that a major site of interaction lies in the C-terminal region. A possible explanation for this discrepancy is that the $G_{\beta\gamma}$-binding function of phosducin's

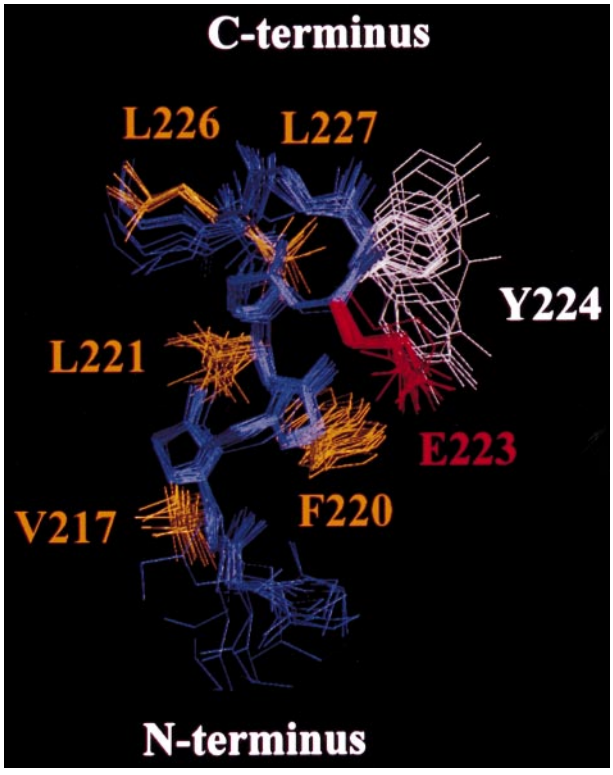

Fig. 7. Superposition of 20 from 80 calculated NMR structures with the lowest energy comprising residues phosducin(215–227). Only the protein backbone (blue), hydrophobic residues (yellow) and the residues E223 (red) and Y224 (white) are shown. These 20 structures fit in the residue range from D216 to Y224 with an average backbone root mean square deviation of 0.5 ± 0.2 Å.

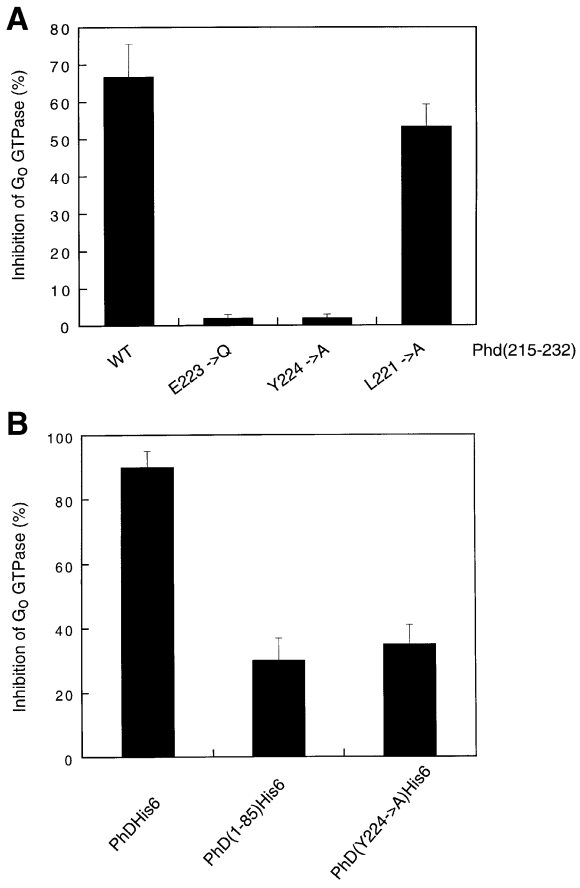

Fig. 8. (**A**) Inhibition of G_o GTPase activity by phosducin(215–232) and variants. The effects of the peptides were assayed as in Figure 2 at a concentration of 30 μM. The inhibition by the same concentration of the unrelated peptide KTAIAKFERLQTVTNYFITSK (see Figure 3) was subtracted as non-specific background. WT: wild-type sequence (see Figure 3). Data are mean ± SEM of three independent experiments. (**B**) Inhibition of G_o GTPase activity by phosducin, phosducin(1–85) and phosducin(Y224→A). The effects of the three His_6-tagged proteins were assayed at a concentration of 1 μM. Data are means ± SEM of three independent experiments.

C-terminus is disturbed when the N-terminal $G_{\beta\gamma}$-binding segment of phosducin is replaced by GST.

Peptides corresponding to this C-terminal segment of phosducin have submicromolar affinity for $G_{\beta\gamma}$, as revealed by their ability to inhibit the GTPase activity of G_o. They are thus reminiscent of peptides derived from adenylyl cyclase-2 (Chen *et al.*, 1995; Weng *et al.*, 1996) which also inhibit $G_{\beta\gamma}$-mediated functions, albeit at concentrations of 10–100 μM. From these peptides and similar sequences present in other $G_{\beta\gamma}$-interacting proteins, a consensus motif N/Q-X-X-D/E-R/K has been proposed for $G_{\beta\gamma}$ binding (Chen *et al.*, 1995). Even though the sequence of phosducin(217–231) contains these amino acids, it lacks the correct spacing to correspond to this consensus motif. This view is supported by the fact that the critical sequence FLNEYGLL has no identical or similar counterpart in any eukaryotic protein contained in GenBank or SwissProt data banks (data not shown). This suggests that the proposed consensus motif for $G_{\beta\gamma}$ binding is not utilized by phosducin, even though the phosducin region has a much higher affinity for $G_{\beta\gamma}$ than peptides containing this proposed consensus motif.

Gaudet *et al.* (1996) recently have completed an X-ray crystallographic structure of a complex containing phosducin and transducin-βγ. The NMR structure of the phosducin peptide is very similar to the corresponding region of phosducin in this complex (Figure 9). The X-ray and the NMR structures fit in the amino acid range from D216 to Y224 with an averaged backbone root mean square deviation of 0.6 ± 0.1 Å. This suggests that the structure of the peptide corresponds to the biologically active conformation and underlines the functional importance of this structure.

The mechanism of $G_{\beta\gamma}$ inhibition by these peptides remains to be unravelled. Specific binding to a single site on $G_{\beta\gamma}$ is indicated by three observations: first, the peptides appear to act directly on G-proteins, since they are active on G_o in solution, precluding an action via disturbance of $G_{\beta\gamma}$–membrane interactions; second, the peptides act only in the presence of $G_{\beta\gamma}$ but not on isolated α_o, suggesting that they act on $G_{\beta\gamma}$; and third, the inhibition curves of the peptides are monophasic with a Hill coefficient of 1, indicating interaction with a single binding site on $G_{\beta\gamma}$. The X-ray structure of the phosducin–transducin-βγ complex shows large interfaces between these proteins, with interactions of $G_{\beta\gamma}$ with the N-terminus as well as the C-terminal domain of phosducin (Gaudet *et al.*, 1996). Our data show that a small C-terminal fragment of phosducin is sufficient to cause interactions of higher affinity than the previously identified N-terminal binding site. Two findings led us to assume that the high-affinity binding of C-terminal phosducin peptides to $G_{\beta\gamma}$ occurs in the same manner as that found for the corresponding region of the intact

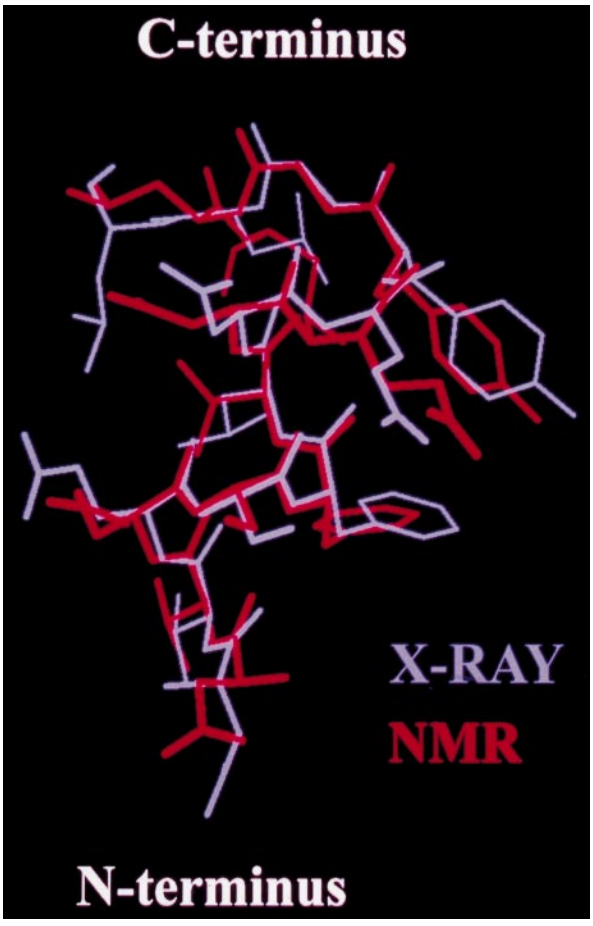

Fig. 9. Superposition of the NMR structure with the lowest energy (red) and the corresponding part of the X-ray structure (blue) comprising the residues phosducin(216–227). The X-ray structure was taken from the phosducin–transducin-βγ complex (Gaudet *et al.*, 1996). The structures fit in the range from D216 to Y224 with an average backbone root mean square deviation of 0.6 ± 0.1 Å.

protein. Firstly, the similarity of the peptide NMR structure to the X-ray structure of the corresponding region in intact phosducin supports an identical mode of binding. Secondly, a mutation (Tyr224→Ala) that inactivates the peptide causes a corresponding inactivation in intact phosducin.

The X-ray structure of the phosducin–transducin-βγ complex locates the C-terminus of phosducin adjacent to the side of blades 1 and 7 of the β-propeller. This site is distinct from the contact sites between β- and α-subunits in heterotrimeric G_{i1} (Wall *et al.*, 1995) or G_t (Lambright *et al.*, 1996). However, these heterotrimeric structures were obtained in the absence of magnesium. Under these conditions, βγ-subunits inhibit GDP release from and thereby GTPase activity of the α-subunits, while at high magnesium concentrations (as used in our experiments) they are stimulatory—probably in a manner similar to their role in receptor-catalysed activation of G-proteins (Higashijima *et al.*, 1987). Since the crystal structures are compatible with inhibitory effects of $G_{\beta\gamma}$, it is likely that there are differences in the mode of contact between G_α and $G_{\beta\gamma}$ in the presence of high magnesium concentrations (or receptors). Therefore, the structural aspects of $G_{\beta\gamma}$ stimulation of the activity of G_α are unknown, and presently it is not possible to delineate in structural terms how the phosducin peptides would effect the inhibition of this $G_{\beta\gamma}$–G_α interaction, in particular whether they block a contact site between G_α and $G_{\beta\gamma}$ or whether they act via structural changes that have been observed upon contact with phosducin in three of the loops of blades 1 and 3 in G_β (Gaudet *et al.*, 1996).

Our studies indicate that in the various assays carried out in the presence of membranes, the $G_{\beta\gamma}$–G_α interaction was ~5-fold more sensitive to the effects of the phosducin peptides than were $G_{\beta\gamma}$–effector interactions (Figure 4). This suggests that G_α and effector molecules may have different modes of binding to $G_{\beta\gamma}$. While some data indicate a common binding mode of the $G_{\beta\gamma}$ effectors adenylyl cyclase-2, PLC-β, β-adrenergic receptor kinase-1 and potassium channels (Chen *et al.*, 1995), other experiments point to distinct binding modes (Yan and Gautam, 1997). Because it lacks the proposed consensus motif and because it probably binds to the side of the β-propeller, the interaction with the C-terminal binding site in phosducin may represent yet another type of interaction with $G_{\beta\gamma}$. Rather than competing for a common binding surface on $G_{\beta\gamma}$ (Weng *et al.*, 1996), this critical sequence contained in phosducin may independently inhibit interactions of G_β with G_α (and, at higher concentrations, with effectors). The small size of this region, together with its defined structural elements, suggests that it might constitute a basis for the development of $G_{\beta\gamma}$ inhibitors.

Materials and methods

Construction and purification of phosducin–His_6 fusion proteins

In order to generate phosducin with a C-terminal His_6 tag, a synthetic cDNA fragment corresponding to a 41 bp *Nsi*I–*Bam*HI fragment at the very C-terminus of phosducin and encoding six additional consecutive histidine residues in front of the stop codon of phosducin was ligated into the corresponding position in the bacterial expression vector pET-Phd (Bauer *et al.*, 1992) containing the coding region of bovine phosducin. N- and C-terminal deletions of phosducin–His_6 were generated via polymerase chain reactions (PCRs) using 5′ and 3′ primers encoding the new phosducin fragments. To construct the phosducin(Y224→A) mutant, a synthetic cDNA fragment corresponding to a 699 bp *Nco*I–*Nsi*I fragment was generated via PCR using a reverse primer carrying the Tyr→Ala mutation in its sequence. This fragment was ligated in the corresponding position in the phosducin His_6 vector (see above). All cDNA constructs were verified by sequencing.

The vectors were introduced into the *Escherichia coli* strain BL21-(DE3)pLysS and induced as described (Bauer *et al.*, 1992). The induced cells were lysed in 50 mM Na-phosphate buffer pH 7.4 by sonication. The lysate was centrifuged at 19 000 *g* for 30 min and the His_6-tagged proteins were purified from the supernatant to >95% homogeneity by chromatography on Ni-NTA columns (Quiagen).

Peptide synthesis

Peptides were synthesized automatically on a MilliGen 9050 peptide synthesizer by the solid-phase method using standard Fmoc chemistry in the continuous flow mode. Final cleavage from the resin and deprotection of side chains was achieved by a mixture of 88% trifluoroacetic acid/5% phenol/5% water/2% tri-isopropylsilane for 3 h, and purification was carried out by preparative HPLC, giving products of >95% purity. Peptides were characterized by matrix-assisted laser desorption/ionization mass spectrometry, which gave the expected [M+H] peaks, and amino acid analysis.

Determination of the GTPase activity of G_o

The steady-state GTPase activity of isolated G_o (purified from bovine brain according to Sternweis and Robishaw, 1984) was measured in solution [10 mM HEPES, pH 7.6, 0.5% (w/v) Lubrol] by determining the release of ^{32}P from [γ-^{32}P]GTP as described earlier (Lohse *et al.*, 1992). Unless indicated otherwise, 0.2 pmol of G_o was assayed in the

presence of 25 mM $MgSO_4$ in a reaction volume of 100 µl. The incubations lasted for 30 min at 30°C and were terminated by addition of 500 µl of 1% charcoal in 2 mM NaH_2PO_4, pH 2. Phosducin–His_6 proteins or phosducin peptides were present at the indicated concentrations, and the concentration–response curves were analysed with the Hill equation as described (Lohse *et al.*, 1986).

The MAS-7-stimulated (Higashijima *et al.*, 1990) GTPase activity of G_o was measured with G_o (0.1 pmol per tube) reconstituted into crude phosphatidylcholine vesicles as described (Lohse *et al.*, 1992). GTPase assays were performed as described above in the presence of 50 µM MAS-7 (Biomol).

Detection of $G_{\beta\gamma}$ binding to His_6-tagged phosducins

To determine their $G_{\beta\gamma}$-binding capability, purified phosducin–His_6 proteins (250 pmol) were incubated with 130 pmol of $G_{\beta\gamma}$ purified from bovine brain in phosphate-buffered saline (PBS) containing 0.05% cholate (140 mM NaCl, 30 mM KCl, 6.5 mM Na_2HPO_4, pH 7.3) for 20 min on ice (Pitcher *et al.*, 1992). The fusion proteins were then bound to 30 µl of Ni-NTA resin (Qiagen). The beads were washed in the same buffer with intervening short centrifugations, and the bound $G_{\beta\gamma}$ was detected by taking up the beads in SDS sample buffer followed by SDS–PAGE and Western blotting with antibodies against the β-subunit (Signal Transduction Laboratories). Peroxidase-coupled secondary antibodies and enhanced chemoluminescence reagents (Amersham) were used to develop the blots.

Adenylyl cyclase assays

Adenylyl cyclase-2-expressing membrane preparations (4–6 mg of membrane protein from Sf9 cells/assay tube) were incubated with GTPγS-activated $rG_{s\alpha}$ (100 nM) for 10 min at 30°C in 20 mM Na-HEPES (pH 8.0), 2 mM $MgCl_2$, and 1 mM EDTA. The different peptides were incubated with purified bovine brain $G_{\beta\gamma}$ (100 nM) for 20 min at 4°C. Adenylyl cyclase activity was measured as described earlier (Pippig *et al.*, 1993). The resulting [^{32}P]cAMP was purified by precipitation of ATP and chromatography on alumina.

Phosphorylation of rhodopsin

Urea-treated rod outer segments containing >95% rhodopsin were prepared from bovine brain retina (Wilden and Kühn, 1982). Rhodopsin (40 pmol) was phosphorylated in a volume of 40 µl using 2.5 nM purified recombinant βARK-1 and 20 nM G-protein βγ-subunits from bovine brain as described earlier (Schröder and Lohse, 1996). Phosducin was present at 100 µM. The incubation was carried out at 30°C for 6 min under bright white light. The reaction was stopped, and the samples were resolved by SDS–PAGE. The absolute determination of ^{32}P content of rhodopsin was done by excising the bands from the gel and Cerenkov counting.

Phospholipase C assays

PLC-β2 activity was determined with a truncated version of PLC-β2 as described by Dietrich *et al.* (1994). [^{3}H]Phosphatidyl 4,5-bisphosphate served as the substrate. When present, the concentration of $G_{\beta\gamma}$ was 600 nM, and that of phosducin (215–232) 100 µM.

NMR experiments and structure calculations

NMR samples contained 2 mM phosducin(204–231), 10 mM Na-phosphate and 100 mM NaCl in 10% D_2O/90% H_2O or in pure D_2O at pH 6.5. The spectra were recorded either on an AMX-500 or an AMX-600 spectrometer (Bruker). All two-dimensional spectra were acquired in phase-sensitive mode (TPPI, Marion and Wüthrich, 1986) with appropriate suppression of the water resonance (WATERGATE pulse sequence, Piotto *et al.*, 1992). Clean TOCSY spectra (Griesinger *et al.*, 1988) were measured using a TOWNY sequence for spin lock (Kadkhodaei *et al.*, 1993). Mixing times used were 60 ms for the TOCSY and 40–250 ms for the NOESY experiments. Two-dimensional spectra were recorded at 277 K, with 2048 data points in the acquisition domain and 768–1024 data points in the t_1 domain. Prior to Fourier transformation, the data were zero-filled to 2048 points in the t_1 dimension, and multiplied with appropriate window functions. Baseline corrections were performed in both dimensions using a polynomial. Structures were generated using 87 sequential, 60 medium range, 13 long range NOEs and five hydrogen bond mimic distances with a standard simulated annealing protocol (Nilges *et al.*, 1993) and an extended version of X-PLOR 3.851 (Brünger, 1993). Eighty structures were calculated starting from conformations with random backbone torsion angles. Nine NOEs per residue were applied in the amino acid range 216–226. The averages of the X-PLOR potential energies found for the 20 structures with the lowest energies were $\langle E_{total} \rangle = 67 \pm 3$, $\langle E_{bonds} \rangle = 2.8 \pm 0.4$, $\langle E_{angles} \rangle = 41 \pm 4$, $\langle E_{impropers} \rangle = 7 \pm 1$, $\langle E_{vdW} \rangle = 9 \pm 1$ and $\langle E_{NOE} \rangle = 9 \pm 1$ kcal/mol. These structures fit with an backbone average root mean square deviation of 0.5 ± 0.2 Å.

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