Phosphorylation of Phosducin and Phosducin-like Protein by G Protein-coupled Receptor Kinase 2*

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G protein-coupled receptor kinase 2 (GRK2) is able to phosphorylate a variety of agonist-occupied G protein-coupled receptors (GPCR) and plays an important role in GPCR modulation. However, recent studies suggest additional cellular functions for GRK2. Phosducin and phosducin-like protein (PhLP) are cytosolic proteins that bind Gβγ subunits and act as regulators of G-protein signaling. In this report, we identify phosducin and PhLP as novel GRK2 substrates. The phosphorylation of purified phosducin and PhLP by recombinant GRK2 proceeds rapidly and stoichiometrically (0.82 ± 0.1 and 0.83 ± 0.09 mol of P/mol of protein, respectively). The phosphorylation reactions exhibit apparent Ki values in the range of 40–100 nM, strongly suggesting that both proteins could be endogenous targets for GRK2 activity.

Our data show that the site of phosducin phosphorylation by GRK2 is different and independent from that previously reported for the cAMP-dependent protein kinase. Analysis of GRK2 phosphorylation of a variety of deletion mutants of phosducin and PhLP indicates that the critical region for GRK2 phosphorylation is localized in the C-terminal domain of both phosducin and PhLP (between residues 204 and 245 and 195 and 218, respectively). This region is important for the interaction of these proteins with Gβγ subunits. Phosphorylation of phosducin by GRK2 markedly reduces its Gβγ binding ability, suggesting that GRK2 may modulate the activity of the phosducin protein family by disrupting this interaction. The identification of phosducin and PhLP as new substrates for GRK2 further expands the cellular roles of this kinase and suggests new mechanisms for modulating GPCR signal transduction.
Fig. 1. Purified phosducin is a substrate for GRK2. A, identification of phosducin as a potential GRK2 substrate. Rat liver plasma membranes (3.4 μg of protein) were preincubated in 50 μl of buffer (20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 50 μM AIF₄⁻, 1 mM GDP) with 20 nM GRK2 for 15 min at 37 °C as described previously (22). The phosphorylation assay was initiated by adding 2 μM rhodopsin and phosphorylation buffer as detailed under “Experimental Procedures” followed by incubation for 20 min at 30 °C in the presence or absence of 600 nM purified phosducin. Phosphorylated proteins were resolved by 10% SDS-PAGE and analyzed by autoradiography. B and C, GRK2 phosphorylates phosducin. Purified phosducin-His₆ (100 nM) was incubated in phosphorylation buffer as detailed under “Experimental Procedures” with 25 nM purified GRK2 for 60 min at 30 °C in the presence or absence of 50 units of PKI or of the GRK2 inhibitor heparin (C, 0.8 μM) as indicated. After stopping the reaction, proteins were resolved by SDS-PAGE and analyzed by autoradiography. The first lane in B shows GRK2 autophosphorylation. D, phosducin is phosphorylated by GRK2 and PKA at different and independent sites. Phosducin-His₆ (100 nM) was preincubated in 25 μl of phosphorylation buffer with unlabeled ATP either with 25 nM GRK2 for 60 min (left) or with 5 μM of PKA and 5 μM cAMP for 10 min (right). Control phosducin aliquots were preincubated under the same conditions without any kinase present. The pre-phosphorylated phosducin was used as a substrate for a subsequent phosphorylation assay with the other kinase. Reactions were initiated by adding 25 μl of phosphorylation buffer including [γ-³²P]ATP to a final specific activity of 4–6 cpm/fmol, and the indicated additions of PKA or GRK2 to the final concentrations that are detailed above. Proteins were resolved and analyzed as in previous panels. E, phosphorylation of phosducin and the S73A phosducin mutant by PKA and GRK2. His₆-tagged wild-type phosducin and a S73A mutant lacking the PKA phosphorylation site (100 nM) was incubated in 25 μl of phosphorylation buffer with unlabeled ATP either with 25 nM GRK2 for 60 min (left) or with 5 μM of PKA and 5 μM cAMP for 10 min (right). Control phosducin aliquots were preincubated under the same conditions without any kinase present. The pre-phosphorylated phosducin was used as a substrate for a subsequent phosphorylation assay with the other kinase. Reactions were initiated by adding 25 μl of phosphorylation buffer including [γ-³²P]ATP to a final specific activity of 4–6 cpm/fmol, and the indicated additions of PKA or GRK2 to the final concentrations that are detailed above. Proteins were resolved and analyzed as in previous panels. F, additive phosphorylation of phosducin by PKA and GRK2. His₆-tagged phosducin (100 nM) was incubated as detailed above in the presence of the indicated kinases, and phosphorylation was analyzed by SDS-PAGE and autoradiography.

expressed (25, 31). The homologous PhLP also shows a broad pattern of expression (28, 29). Both proteins have been reported to compete for Gβγ binding with other targets such as adenyl cyclases or phospholipase Cβ2 resulting in modulation of GPCR signaling (25, 29, 30, 32–34). In the case of phosducin, its ability to interact with Gβγ dimers is attenuated upon phosphorylation by the cAMP-dependent protein kinase (PKA), thus providing a mechanism for regulating its function (35, 36), whereas regulation of PhLP remains unclear because PKA does not phosphorylate PhLP (37).

Despite this data, very little is known about how these regulatory mechanisms at the receptor and G-protein levels are integrated during cellular responses to agonists. Because both GRK2 and phosducins are able to interact with Gβγ subunits, it is tempting to suggest that they may influence one another. In fact, phosducins have been shown to inhibit Gβγ-mediated GRK2 activation and translocation (29, 38). In this report, we show that phosducin and PhLP are high-affinity substrates for GRK2 and that such phosphorylation results in inhibition of the Gβγ binding ability. Together with recent data that identify tubulin as a substrate for GRK2 (39, 40), our results corroborate the existence of non-GPCR substrates for this kinase and suggest a key role for GRK2 in the modulation of GPCR signaling at different levels.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—**GRK2, GRK3, and GRK5 were overexpressed and purified from baculovirus-infected S9 cells as described previously (22, 41). C-terminal hexahistidine-tagged wild-type phosducin (phosducin-His₆), a S73A mutant lacking the PKA phosphorylation site, and several N- and C-terminal truncated variants (phosducin-(1–204), phosducin-(64–245), and phosducin-(138–245)) were expressed as glutathione S-transferase fusion proteins (GST-PhLP) as described previously (42). Gβγ proteins were purified from bovine brain as reported (43).

**Phosphorylation Assays—**GRK2 phosphorylation of phosducin-His₆, GST-PhLP, and the truncated constructs was performed in 50 μl of 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM EDTA, 1.4 mM EGTA, 25–60 μM ATP (2–3 cpm/fmol) (phosphorylation buffer), and the desired concentrations of GRK2, phosducin, or PhLP as indicated in the figure legends. In some experiments, 1 mM dithiothreitol, 10% glycerol, 0.8 μM heparin (Sigma) or 100 units of the peptide PKA inhibitor PKI (Sigma) were included in the assay buffer. After incubation for the desired time at 30 °C, the reaction was stopped by adding 25 μl of SDS-sample buffer and heating for 5 min at 95 °C. Samples were resolved by 10–12.5% SDS-PAGE except for the C-terminal truncated constructs of phosducin that were resolved by using a 16.5/Tricine-SDS gel. The phosphorylated proteins were detected by autoradiography. Cerenkov counting of the excised bands was used to quantitate the phosphorylation and determine stoichiometry.

To compare the capacity of GRK2, GRK3, and GRK5 to phosphoryl-
ate phosducin, each GRK was dialyzed in salt-free phosphorylation buffer for 12 h just before usage to exclude the potential interference of different buffer conditions on phosducin phosphorylation. Aliquots of the GRK dialysates were used in a rhodopsin phosphorylation assay as described previously (38) to assess its activity, and equally effective amounts were then used in the phosducin phosphorylation assays.

For PKA-mediated phosphorylation assays, 25–100 nM of purified phosducin-His$_6$ was incubated for 20 min at 30 °C in the same phosphorylation buffer used with GRK2 in the presence of 5–10 units of PKA and 5 μM cAMP (Sigma) in a final volume of 50 μl in the presence or absence of 80 units of PKI. Phosphorylation assays were stopped, resolved, and analyzed as detailed above. In some experiments, phosducin was subjected to phosphorylation by either PKA or GRK2 in the absence of radiolabeled ATP prior to its incubation with the other kinase in the presence of [γ-32P]ATP, to investigate whether these were independent processes (see legend to Fig. 1).

Two-dimensional Gel Electrophoresis—To analyze the number of Ser/Thr residues in phosducin and PhLP that were phosphorylated by these were independent processes (see legend to Fig. 1).

Identification of Phosducin as a GRK2 Substrate—We have previously described that GRK2 binds to intracellular membranes under basal conditions by means of an as yet unidentified anchoring protein and that this interaction inhibits its phosphorylation activity (21, 22). Several lines of evidence suggest that membrane-bound GRK2 activity could be enhanced by stimulation of endogenous heterotrimeric G proteins with aluminum fluoride (22). Because phosducin has been described as an inhibitor of Gβγ-stimulated GRK2 activity toward agonist-activated receptors by competing for binding with free Gβγ subunits (31, 34), purified bovine phosducin was included in our assays to test the participation of endogenous Gβγ subunits in the observed stimulation of GRK2 activity. As expected, phosducin (600 nM) did inhibit light-dependent phosphorylation of rhodopsin (2 μM) by GRK2 (Fig. 1A). However, it was noted that a band of ~33 kDa was heavily phosphorylated under these conditions (Fig. 1A, lane 2). This band co-migrated with purified phosducin and was recognized by antibodies raised against purified recombinant phosducin (Ref. 31, data

**RESULTS**

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Procedures.

GST was incubated with 25 nM GRK2 in phosphorylation buffer for 5 min at 30 °C, and the extent of phosphorylation was quantified as above. Data are mean ± S.E. of three independent experiments. A representative experiment is shown. C, two-dimensional gel electrophoresis analysis of phosphorylated GST-PhLP. GST-PhLP (25 nM) was incubated with 25 nM GRK2 in phosphorylation buffer for 15 min at 30 °C. The reaction was stopped by adding isoelectric focusing buffer, and the sample was processed as indicated under “Experimental Procedures.”

not shown). To test the possibility that GRK2 was the kinase directly responsible for the observed phosducin phosphorylation, we performed additional experiments using recombinant-purified GRK2 and hexahistidine-tagged phosducin. As shown in Fig. 1B, phosducin was markedly phosphorylated in the presence of GRK2. Additional experiments were carried out to confirm that this phosphorylation was a result of GRK2 activity and could not be attributed to contaminating PKA, which might be present at very low concentrations in the purified GRK2 preparation. The addition of high concentrations of the PKA inhibitor heparin as indicated. Proteins were resolved by SDS-PAGE and analyzed by autoradiography. An autoradiogram representative of three experiments is shown.

Characterization of Phosducin Phosphorylation by GRK2—Fig. 2A shows that phosducin phosphorylation by GRK2 proceeds rapidly (t1/2 = 15 min), stoichiometrically (0.82 ± 0.1 mol of P/mol of phosducin, when both phosducin and GRK2 are present at 25 nM during the phosphorylation assay), and is dependent on phosducin concentration (Fig. 2B). Double-reciprocal plot analysis (Fig. 2C) reveals that GRK2 displays an apparent Km for phosducin of 46 ± 7 nM, with a Vmax of 0.72 ± 0.04 nmol P/min/mg of protein. The observed affinity is well below the physiological range (−1 µM) of phosducin concentrations (31) and similar or higher than that reported for other GRK2 substrates (39, 40, 47). Finally, in agreement with the stoichiometric data, two-dimensional gel electrophoresis analysis of GRK2-phosphorylated phosducin indicates that most of time (60 and 10 min, respectively) known to allow the completion of these reactions (data not shown). Control phosducin aliquots were incubated in the same buffer conditions in the absence of any kinase. Next, the pre-phosphorylated phosducin was tested as a substrate for the other kinase by including purified PKA or GRK2 in the reaction together with [γ-32P]ATP. As shown in Fig. 1D, prior phosducin phosphorylation by GRK2 had no effect on PKA activity toward phosducin (left), nor did phosphorylation by PKA significantly alter the subsequent phosphorylation by GRK2 (right). The fact that the observed phosphorylation is not due to remaining activity of the first kinase was confirmed by the absence of radioactive labeling observed upon addition of [γ-32P]ATP when no additional kinase was present. To further demonstrate that GRK2 and PKA phosphorylate phosducin at different sites, we tested the ability of GRK2 to phosphorylate a phosducin mutant (S73A) that lacks the phosphorylation site for PKA (35). Fig. 1E shows that wild-type phosducin and the S73A mutant were phosphorylated to a similar extent by GRK2 whereas PKA phosphorylation was completely abolished in the mutant. Moreover, simultaneous incubation with PKA and GRK2 led to an additive incorporation of phosphate (Fig. 1F). Overall, these data indicate that phosducin phosphorylation by both kinases is not mutually exclusive and takes place at different residues, suggesting that PKA and GRK2 may be independent modulators.

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Phosducin Phosphorylation by GRK2

Each phosducin-His6 construct (containing the indicated amino acids) of different phosducin constructs by GRK2. 25 nM (data not shown). Time-course analysis (Fig. 3) revealed that all phosphorylation is unclear (37). Therefore, we tested the ability of GRK2 to phosphorylate a variety of purified phosducin and PhLP constructs in which different domains of these proteins had been deleted. The N-terminal truncated phosducin construct phosducin-(64–245) was efficiently phosphorylated by GRK2 (Fig. 5A, panel II). The phosducin-(138–245) deletion construct was clearly phosphorylated by GRK2 (Fig. 5B). We then explored whether phosducin was a specific substrate for GRK2 or whether it could also be phosphorylated by other members of the GRK family. To address this issue, aliquots of GRK2, GRK3, and GRK5 that were equally effective in phosphorylating rhodopsin (data not shown) were tested for their activity toward purified phosducin. Fig. 4 shows that all tested GRKs were able to phosphorylate phosducin although with slightly different activities.

Localization of Phosphorylation Sites and Functional Consequences of Phosducin Phosphorylation by GRK2—We next attempted to localize the region of phosducin and PhLP that contains the site(s) of phosphorylation by GRK2. For this purpose, we tested the ability of GRK2 to phosphorylate a variety of purified phosducin and PhLP constructs in which different domains of these proteins had been deleted. The N-terminal truncated phosducin construct phosducin-(64–245) was efficiently phosphorylated by GRK2 (Fig. 5A, panel II). The phosducin-(138–245) deletion construct was clearly phosphorylated by GRK2 (Fig. 5A, panel II), although it displayed a ~2.5-fold increase in the phosphorylation K_m with respect to full-length phosducin (data not shown). In contrast, phosducin-(1–204) was poorly phosphorylated by GRK2 even in the presence of a higher GRK2 concentration (Fig. 5A, panel II). Over-all, our results indicate that the C-terminal region of phosducin (residues 205–245) contains the main determinants required for GRK2 phosphorylation (the phosphorylation site itself and/or docking regions critical for the action of the kinase). Similar experiments with a variety of GST-PhLP constructs (Fig. 5B) also showed that the region essential for phosphorylation by GRK2 resides in the C-terminal region of the protein.
Phosducin Phosphorylation by GRK2

Whereas the PhLP-(143–218) and PhLP-(168–218) constructs were efficiently phosphorylated by GRK2, deletion of only the last 23 residues in the C-terminal domain (PhLP-(1–195)) completely abolished PhLP phosphorylation by GRK2.

The C-terminal region of both phosducin and PhLP have been shown to play a critical role in their interaction with Gβ subunits (26, 27, 30, 42). The fact that these regions are also critical for GRK2 phosphorylation suggests that the activity of this kinase may modulate the ability of phosducin to bind Gβγ dimers. To test this hypothesis, we investigated the effect of preincubating phosducin with GRK2 under phosphorylating or non-phosphorylating conditions on the subsequent interaction of histidine-tagged phosducin with purified brain Gβγ subunits. After removal of GRK2 with Sephaphore, the ability of unphosphorylated (no ATP) or GRK2-phosphorylated (+ATP) phosducin to interact with two different concentrations of Gβγ subunits was assessed (Fig. 6A). A marked reduction in Gβγ binding to phosphorylated phosducin (~50%, Fig. 6B) was noted at 50 nM Gβγ, whereas such a decrease was not apparent at high Gβγ concentrations (1000 nM), thus suggesting that GRK2 phosphorylation decreases the affinity of the Gβγ-phosducin interaction. Whereas Gβγ subunits did not bind to Ni2+/-NTA-agarose on its own, GRK2 and its Gβγ binding ability were still detectable in the background control (Fig. 6A). However, the signals were much weaker than those observed in the presence of phosducin and were visible only at high concentrations of Gβγ (1000 nM).

**DISCUSSION**

In this report, we have identified phosducin and PhLP as new substrates for GRK2. In line with recent reports that show that tubulin is phosphorylated by this kinase (39, 40), our data further indicate the presence of soluble substrates for GRK2. The emerging evidence that the activity of this kinase is not restricted to agonist-occupied GPCR suggests that GRK2 may also act as an effector of GPCR signaling and have additional cellular roles.

Several lines of evidence support the hypothesis that GRK2-mediated phosphorylation of phosducin proteins may have physiological relevance. The phosphorylation reactions proceed rapidly and stoichiometrically. The apparent Km (40 nM for phosducin and 103 nM for PhLP) displays far higher affinity than that observed for peptide substrates of GRK2 (10,000-fold) than that reported for peptide substrates of GRK2 (39, 40), suggesting that GRK2-mediated phosphorylation may represent an additional mechanism for the modulation of phosducin interaction with Gβγ subunits released upon receptor-mediated G or Gα activation. GRK2-mediated regulation of phosducins further differs from that by PKA in three important ways. (a) The phosphorylation of phosducin by PKA and GRK2 is independent and takes place at different sites. (b) Although the effect of PKA is restricted to phosducin, GRK2 also phosphorylates PhLP. These data suggest independent control of phosducin functions by the two different types of kinases.

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