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Altered Activity of Palmitoylation-deficient and Isoprenylated Forms of the G Protein-coupled Receptor Kinase GRK6*

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G protein-coupled receptor kinases (GRKs) utilize diverse mechanisms to associate with the plasma membrane and mediate phosphorylation of agonist-occupied receptors. For example, two members of this family, GRK4 and GRK6, contain C-terminal cysteine residues that are palmitoylated. To address whether the activity and membrane association of GRK6 is regulated by palmitoylation, we overexpressed and characterized wild-type GRK6 and two GRK6 mutants, one with the palmitoylation sites mutated to serines (GRK6-pal$^-$) and one containing a C-terminal CAAX motif to promote geranylgeranylation (GRK6-GG). Compared with wild-type GRK6, GRK6-pal$^-$ had a ~5-fold higher K_m and ~2-fold lower V_{max} for phosphorylating rhodopsin, whereas GRK6-GG exhibited a ~2-fold lower K_m and ~14-fold higher V_{max} for rhodopsin. In contrast, wild-type GRK6 and GRK6-pal$^-$ displayed similar activity toward the nonreceptor substrate phosvitin, indicating that nonpalmitoylated GRK6 is catalytically active. Wild-type GRK6 and GRK6-GG, but not GRK6-pal$^-$, also bound significantly to phosphatidylcholine vesicles (36 ± 3, 79 ± 4, and 4 ± 2%, respectively) suggesting that GRK6 activity is dependent upon its ability to interact with the plasma membrane. When assayed in COS-1 cells GRK6-pal$^-$ promoted minimal agonist-dependent sequestration of the β_2-adrenergic receptor, while sequestration was significantly increased in cells expressing either wild-type GRK6 or GRK6-GG. These data demonstrate an important functional link between the ability of GRK6 to bind to the plasma membrane, a process that appears to be regulated by palmitoylation, and its activity toward receptor substrates.

G protein-coupled receptors mediate numerous intracellular signaling pathways upon binding extracellular agonists (*e.g.* hormones, neurotransmitters, odorants, chemoattractants, and light) (1, 2). Receptors of this type regulate a variety of effector molecules such as adenylyl cyclase, cGMP phosphodiesterase, phospholipases A_2 and C, and numerous ion channels. Two of the best studied G protein-coupled receptors are the β_2-adrenergic receptor (β_2AR),[1] which mediates catecholamine stimulation of adenylyl cyclase, and the visual light receptor, rhodopsin, which facilitates phototransduction in retinal rod cells (3, 4). In both systems a rapid diminution of responsiveness or desensitization occurs following receptor activation (1, 3–5). Activation-dependent desensitization is mediated in part by specific G protein-coupled receptor kinases (GRKs) that have the unique ability to recognize and phosphorylate their receptor substrates only when they are in an active conformation (6, 7). The β-adrenergic receptor kinase (βARK) and rhodopsin kinase have been implicated as the major kinases involved in the stimulus-dependent phosphorylation of the β_2AR and rhodopsin, respectively (8–11). Subsequent uncoupling of the receptor from the G protein is promoted by arrestin proteins that specifically bind to the phosphorylated and activated form of the receptor (3–5).

Recent evidence has demonstrated that GRKs employ a variety of mechanisms that promote their localization to the cell membrane (12–19). Rhodopsin kinase, βARK, and βARK2 undergo stimulus-dependent translocation from the cytosol to the plasma membrane, although this is achieved through two somewhat different mechanisms (13, 20). Rhodopsin kinase contains a "CAAX" motif at its C terminus that directs the attachment of a C_{15} isoprenoid (farnesyl) moiety required for membrane binding and optimal kinase activity (12). Agonist-dependent translocation of βARK and βARK2 does not involve direct acylation of these kinases but, instead, appears to be facilitated by their binding to G protein βγ subunits that are themselves membrane-bound via isoprenylation (13). βARK and βARK2 contain a pleckstrin homology (PH) domain within the kinase C terminus that is responsible for the binding of these proteins to phospholipids and $G_{\beta\gamma}$ subunits (13–15, 21). Furthermore, binding of βARK and βARK2 to phospholipids and Gβγ augments kinase activity toward receptor substrates indicating an important regulatory function of this complex in GRK-mediated receptor phosphorylation (14, 15, 22). GRK5, which is not acylated, possesses a highly basic C-terminal domain that enables the kinase to bind to the plasma membrane (17, 23). Interaction of GRK5 with membrane phospholipids enhances kinase autophosphorylation, which increases its activity toward receptor substrates (17).

Studies performed in our laboratory with GRK6 overexpressed in and purified from Sf9 insect cells revealed that GRK6 has significantly lower activity than βARK and GRK5 toward rhodopsin, the β_2AR, and the m2 muscarinic acetylcholine receptor *in vitro* (24). We postulated that the observed differences in GRK6, βARK, and GRK5 activity may be due either to distinct substrate specificities among these GRKs and/or a lack of a functionally relevant cofactor for GRK6. Recently, it was demonstrated that GRK4 and GRK6 are

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[1] The abbreviations used are: β_2AR, β_2-adrenergic receptor; βARK, β-adrenergic receptor kinase; G protein, guanine nucleotide-binding protein; GRK, G protein-coupled receptor kinase; PH, pleckstrin homology; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PC, phosphatidylcholine; PIP_2, phosphatidylinositol 4,5-diphosphate.

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palmitoylated enabling these enzymes to associate with the plasma membrane (18, 19). However, the role that palmitoylation plays in the function of these kinases remains obscure. In an effort to better understand the role that palmitoylation might play in regulating GRK6 localization and activity, we generated a mutant kinase lacking the putative palmitoylation sites and assessed its function both *in vitro* and in intact cells. We show that nonpalmitoylated GRK6 exhibits significantly diminished activity *in vitro* toward rhodopsin compared with wild-type GRK6 and that this reduced activity is likely due to its inability to interact with phospholipid. In contrast, when the palmitoylation-deficient GRK6 is modified with a C-terminal isoprenoid moiety both the activity and phospholipid binding are enhanced compared with wild-type GRK6. Furthermore, we demonstrate that COS-1 cells transiently coexpressing the β_2AR and nonpalmitoylated GRK6 undergo significantly less agonist-dependent receptor sequestration compared with cells expressing either wild-type or isoprenylated GRK6.

EXPERIMENTAL PROCEDURES

Materials—The chromatography resin SP(HP)-Sepharose was purchased from Pharmacia Biotech Inc. Frozen bovine retinas were from George A. Hormel & Co. COS-1 monkey kidney cells were from the American Type Culture Collection. Phosvitin was from Sigma, whereas [γ-^{32}P]ATP was from NEN Life Science Products. Affinity-purified rabbit polyclonal antibody specific for GRK6 and control peptide containing amino acids 525–544 of GRK6 were purchased from Santa Cruz Biotechnology, Inc.

Generation of GRK6 Mutants—Mutant GRK6 sequences were PCR-amplified from the GRK6 cDNA (25) in pBluescript using a forward primer corresponding to bases 1192–1209 (5′-AGATGATCGCAGGCCAGT-3′) and one of two mutant reverse primers corresponding to the 3′ end of the GRK6 sequence: 5′-CAATGGATCCCTAGAGGCGGGTGGGCAGCTCTTCCTCGCTGTCGCTGCTGTTTCCGCTGCTATCTTGGCGACTGA-3′ (GRK6-pal$^-$, palmitoylation-deficient mutant with Cys^{561}, Cys^{562}, and Cys^{565} mutated to serine); or 5′CAATGGATCCCTACAGCAGCACGCAGAGGCGGGTGGGCAGCTCTTCCTCGCTGTCGCTGCTGTTTCCGCTGCTATCTTGGCGACTGA-3′(GRK-6GG, palmitoylation deficient mutant with Cys-Val-Leu-Leu added to the C terminus). The reverse primers also mutagenize the *Sac*I restriction site found at base pair 1777 of the open reading frame to facilitate subcloning into the mammalian expression vector pBC12BI (see below). All PCR reactions were performed using the Expand PCR system (Boehringer Mannheim). PCR products were digested with *Sph*I and *Bam*HI, which yielded ~360-base pair fragments and then subcloned into pBluescript-GRK6 digested with the same enzymes. The PCR derived portion was sequenced using an automated DNA sequencer. DNA was cut with *Sac*I and *Bam*HI restriction enzymes, and the ~1600-base pair fragments subcloned into pBC-GRK6 digested with the same enzymes (26). Oligonucleotides and DNA sequencing were provided by the Kimmel Cancer Institute DNA Facility.

Expression, Purification, and Western Blot Analysis of Wild-type and Mutant GRK6 from Transiently-transfected COS-1 Cells—To overexpress wild-type and mutant GRK6, 12 μg each of pBC-GRK6, pBC-GRK6-pal$^-$, and pBC-GRK6-GG were used to transiently transfect COS-1 cells in T75 tissue culture flasks by the lipofectAMINE method following the manufacturer's instructions (Life Technologies, Inc.). Cells were trypsinized 48 h after transfection, washed several times with ice-cold PBS, and then lysed in 300 μl of 20 mM HEPES, pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, and 200 μg/ml benzamidine. Lysates were centrifuged at 15,000 $\times$ *g* for 5 min at 4 °C and supernatants recovered. Supernatants were diluted to 1.2 ml in 20 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 200 μg/ml benzamidine, and 20 μg/ml leupeptin (buffer A), and GRK6 purification was performed by batchwise SP-Sepharose chromatography using 150 μl of resin. After 1 h of incubation of the resin with the cell lysates at 4 °C on a rotator, the resin was washed six times with 1 ml of buffer A containing 150 mM NaCl to remove trace amounts of βARK present in the adsorbed lysate. A GRK6-enriched fraction was obtained by eluting the resin three times with 200 μl of buffer A containing 400 mM NaCl and pooling the eluants. Quantitation of GRK6 expression in COS-1 cells and recovery after SP-Sepharose chromatography was done by immunoblotting with a polyclonal rabbit antiserum raised against a glutathione *S*-transferase fusion protein corresponding to the C-terminal 102 amino acids of GRK5 (immunoreactive with both GRK5 and GRK6). Equivalent amounts of lysates and partially purified GRK6 preparations (between 5 and 40 μg of total protein measured by Bio-Rad protein assay using bovine serum albumin) were electrophoresed on a 10% SDS-polyacrylamide gel (27), transferred to nitrocellulose, washed in 0.05% Tween, Tris-buffered saline (TTBS), pH 7.5, and blocked for 1 h in TTBS with 5% (w/v) dried nonfat milk. Filters were immunoblotted for 1 h with the polyclonal antiserum (1:100 dilution) in 5% milk, TTBS and then washed five times, 10 min each, with TTBS and incubated with affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) in 5% milk, TTBS (1:4000 dilution). Filters were washed five times in TTBS and visualized by enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, United Kingdom). Wild-type and mutant GRK6 were quantitated by comparing different amounts of the eluted preparations with known amounts of purified GRK6 (1–4 ng) (24). Typical yields from one flask of confluent COS-1 cells were 400–500 ng for wild-type GRK6, 1200–1500 ng for GRK6-pal$^-$, and 150–200 ng for GRK6-GG.

Rod Outer Segment Preparations and Assays for GRK6 Activity—Urea-treated rod outer segments containing rhodopsin were prepared from bovine retinas, and rhodopsin phosphorylation was performed as described previously (11, 24). For time course assays, 1.3 ng of each kinase preparation (determined by quantitative immunoblotting) was incubated with 200 μM [γ-^{32}P]ATP (1 cpm/fmol), 4.6 mM $MgCl_2$, 6.8 μM rhodopsin, 20 mM HEPES, pH 7.5, and 2 mM EDTA in a total volume of 30 μl for 0, 2.5, 5, 10, and 20 min at 30 °C. Reactions were terminated at the indicated times with 15 μl SDS sample buffer, and 30 μl of each sample were electrophoresed on a 10% SDS-polyacrylamide gel. Gels were dried, and autoradiography was performed for 2–3 h at −80 °C. Quantitation was performed by excising the phosphorylated rhodopsin bands and counting in a scintillation counter. Determination of K_m and V_{max} values was performed by assaying 0.1–20 μM rhodopsin with 1.3 ng of the different GRK6 proteins in 30 μl of buffer (200 μM [γ-^{32}P]ATP (1 cpm/fmol), 4.6 mM $MgCl_2$, 20 mM HEPES, pH 7.5, and 2 mM EDTA) for 5 min at 37 °C. Reactions were terminated with 15 μl of SDS sample buffer and processed as described above. In all experiments, wild-type and mutant GRK6 preparations were stored at 4 °C and were used within 3–4 days after purification. No reduction in kinase activity was observed during this time. When tested, SP-Sepharose eluants from pBC12BI-transfected control cells exhibited no detectable levels of rhodopsin phosphorylation over time (data not shown).

Immunoprecipitation and Characterization of GRK6 Activity Toward Phosvitin—5–10 $\times$ 10^6 COS-1 cells transfected with pBC12BI, pBC-GRK6, or pBC-GRK6-pal$^-$ were trypsinized and lysed in 100 μl of 1% Triton X-100 lysis buffer as described above. 3 μg of affinity-purified rabbit polyclonal antiserum (Santa Cruz Biotechnology, Inc.) was coupled to 10 μl of protein A-agarose beads (Boehringer Mannheim), washed three times with buffer B (0.2% Triton X-100, 150 mM NaCl, 20 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 200 μg/ml benzamidine, 20 μg/ml leupeptin), and then incubated with 90 μl of cell lysate for 1 h at 4 °C on a rotator. The remaining 10 μl of lysate was used for Western blot analysis to quantitate the levels of GRK6 expression. The beads were washed three times with buffer B followed by a single wash with 20 mM HEPES, pH 7.5, 2 mM EDTA to remove Triton X-100 and NaCl (both of which inhibit GRK activity), resuspended in 30 μl assay buffer (200 μM [γ-^{32}P]ATP (1 cpm/fmol), 4.6 mM $MgCl_2$, 133 μg/ml phosvitin (Sigma), 20 mM HEPES, pH 7.5, 2 mM EDTA), and incubated for 15 or 20 min at 30 °C. Reactions were terminated with 20 μl of SDS sample buffer, and the samples were centrifuged briefly and then electrophoresed on a 10% SDS-polyacrylamide gel. Gels were dried, and autoradiography was performed. Quantitation of the phosphorylated phosvitin bands was performed as described above for the rhodopsin phosphorylation assays. Recovery of kinase immunoprecipitated from cell lysates containing either wild-type or mutant GRK6 was ~80% (data not shown). Immunoprecipitatable activity from lysates of cells transfected with the pBC vector alone revealed no significant level of phosvitin phosphorylation compared with either wild-type- or GRK6-pal$^-$-expressing cells (data not shown). Furthermore, immunoprecipitation of GRK6 in the presence of a GRK6 peptide corresponding to the kinase C terminus reduced phosvitin phosphorylation to control levels (data not shown).

Phosphatidylcholine Vesicle Binding Assays—Crude soybean phosphatidylcholine (PC) was sonicated on ice at a concentration of 17 mg/ml in 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA. The sonicated PC vesicles were aliquoted and stored at −80 °C, and a new aliquot was used for each experiment. Binding assays were performed using ~15 ng of partially purified wild-type GRK6, GRK6-pal$^-$, and GRK6-GG that

was diluted in 48 μl of buffer containing 20 mM Tris, pH 8.0, 2 mM $MgCl_2$, 150 mM NaCl and pre-spun at 100,000 $\times$ *g* for 10 min at 4 °C to remove any aggregated GRK6. The supernatants (54 μl) were then added to either 6 μl of PC vesicles (to give a final phospholipid concentration of 1.7 mg/ml) or 6 μl of PC buffer alone and incubated at 30 °C for 5 min. The samples were centrifuged at 100,000 $\times$ *g* for 10 min and the supernatants (60 μl) and pellets (resuspended in 60 μl of buffer) were dissolved in SDS sample buffer. 30 μl of each sample was electrophoresed on a 10% SDS-polyacrylamide gel, and the proteins were transferred to nitrocellulose for Western blot analysis as described above. Quantitation of the percent GRK6 bound to PC vesicles was done using a Molecular Dynamics personal densitometer and ImageQuant software.

Receptor Sequestration Assays—COS-1 cells grown to ~90% confluence in T75 flasks were cotransfected for 48 h as described above with 8 μg of pBC-β_2AR and 8 μg of pBC12BI, pBC-GRK6, pBC-GRK6-pal$^-$, or pBC-GRK6-GG. Transfected cells were trypsinized, washed several times with PBS, and resuspended in 1.1 ml of PBS, 0.1 mM ascorbate. 0.5-ml aliquots of each cell suspension were incubated with or without 10 μM (−)-isoproterenol for 0–45 min at 37 °C. Reactions were stopped with ice-cold PBS; the cells were centrifuged at 2,000 $\times$ *g* for 5 min, washed twice with PBS, and then resuspended in 0.5 ml of PBS. Cell surface β_2AR levels were determined by incubating the cells with 10 nM (−)-[^{3}H]-CGP12177 for 3 h at 14 °C followed by vacuum filtration as described (28). Nonspecific binding was determined in the presence of 20 μM alprenolol.

RESULTS

Expression, Purification, and Activity of Wild-type and Palmitoylation-deficient Mutant GRK6—To assess the role of palmitoylation in GRK6 function we used two expression constructs containing either the wild-type GRK6 cDNA (26) or a mutant GRK6 in which the three putative palmitoylated cysteines (Cys^{561}, Cys^{562}, and Cys^{565}) (18) were changed to serines (GRK6-pal$^-$) (Fig. 1*A*). Direct experimental evidence that one or more of these residues is palmitoylated comes from [^{3}H]palmitate labeling of COS cells expressing either wild-type or pal$^-$ GRK6 in which wild-type GRK6, but not the pal$^-$ kinase, incorporated radiolabeled palmitate (18). COS-1 cells transiently expressing wild-type GRK6 or GRK6-pal$^-$ were lysed and the kinases were partially purified by SP-Sepharose chromatography. Partial purification of the expressed proteins was necessary since their activity in crude lysates was largely inhibited due to the detergent in the lysis buffer and inhibitors present in the cell lysates (data not shown). Expression of wild-type GRK6 (~67 kDa) was ~8-fold higher compared with endogenous levels observed in pBC control-transfected cells, whereas the GRK6-pal$^-$ mutant was expressed ~3-fold higher than wild-type GRK6 (Fig. 1*B*, *lanes 1*, *2*, and *4*).

One relatively simple way to assess GRK activity toward G protein-coupled receptors involves the use of bovine rod outer segments that contain high levels of the photoreceptor rhodopsin. Using equivalent amounts of the two GRK6 proteins, time-course studies revealed a ~5-fold reduced ability of GRK6-pal$^-$ to phosphorylate light-activated rhodopsin compared with wild-type GRK6 (Fig. 2*A*). GRK6-pal$^-$ exhibited a significantly lower affinity for rhodopsin as evidenced by a ~5-fold higher K_m (12.0 ± 0.5 μM) compared with wild-type GRK6 (2.6 ± 0.4 μM) (Table I). However, mutation of the GRK6 palmitoylation site modestly reduced (~2-fold; Table I) its apparent V_{max} with respect to wild-type GRK6 suggesting that lack of palmitoylation does not impair kinase catalytic activity. The K_m of GRK6-pal$^-$ is strikingly similar to the K_m for rhodopsin previously reported for Sf9 cell expressed and purified GRK6 (24). This suggests that the purified GRK6 is not palmitoylated, which may contribute to its apparent low activity.

Overexpression, Purification, and Activity of an Isoprenylated Form of GRK6—Since membrane targeting appears important for rhodopsin kinase, βARK, and GRK5 activity (12, 13, 17) and lack of palmitoylation significantly reduces GRK6 phosphorylation of rhodopsin, we next wanted to test the hypothesis that GRK6 activity toward receptor substrates is strongly influenced by its ability to interact with phospholipids. To do this we made a mutant that was still defective in palmitoylation but was also modified to include a C-terminal CAAX motif to promote protein geranylgeranylation (Fig. 1*A*) (29). In this way, we wanted to determine whether the reduced ability of GRK6-pal$^-$ to phosphorylate receptors could be overcome by enhancing its association with phospholipids by isoprenylation. Expression of this protein (GRK6-GG) was approximately 30–40% of that observed for wild-type GRK6 (Fig. 1*B*, *lanes 2* and *6*).

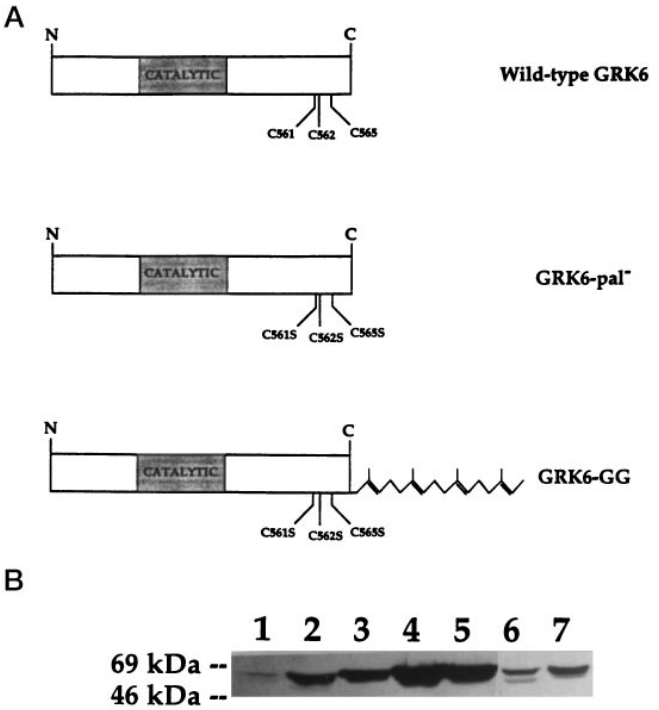

FIG. 1. *Panel A*, diagram of wild-type, palmitoylation-deficient mutant (*GRK6-pal*$^-$) and geranylgeranylated GRK6-pal$^-$ (*GRK6-GG*) showing the overall organization and putative palmitoylation sites. Geranylgeranylated GRK6-pal$^-$ contains a specific "CAAX" motif (CVLL) at the C terminus which gives rise to the isoprenylated protein. *Panel B*, overexpression and purification of wild-type and mutant GRK6. Wild-type and mutant GRK6 constructs were prepared, transiently overexpressed in COS-1 cells, and partially purified as described under "Experimental Procedures." GRK6 expression and recovery was assessed by Western blot analysis using a polyclonal rabbit antiserum that recognizes GRK6. The samples are either crude cell lysates of control, wild-type GRK6, GRK6-pal$^-$, and GRK6-GG (*lanes 1*, *2*, *4*, and *6*, respectively) or SP-Sepharose-purified wild-type GRK6, GRK6-pal$^-$, and GRK6-GG (*lanes 3*, *5*, and *7*, respectively).

When expressed in COS-1 cells and partially purified, GRK6-GG promoted a ~13-fold higher level of rhodopsin phosphorylation compared with wild-type GRK6 (Fig. 2*B*). Furthermore, GRK6-GG had a K_m for rhodopsin of 1.2 μM (2.2-fold lower than wild-type GRK6) and a V_{max} ~14-fold higher than wild-type GRK6 (Table I). These data suggest that GRK6 activity toward a receptor substrate is strongly influenced by its ability to bind to phospholipid membranes.

Activity of Wild-type and Mutant GRK6 Toward Phosvitin—The modest (~2-fold) difference in V_{max} values for wild-type and GRK6-pal$^-$ suggests that mutating the GRK6 palmitoylation site does not significantly affect catalytic activity. To confirm this point we immunoprecipitated wild-type GRK6 and GRK6-pal$^-$ from COS-1 cell extracts and assessed their activity toward a nonreceptor substrate in the absence of phospholipid (26). Previous studies have demonstrated that phosvitin serves as a good *in vitro* substrate for GRK6 (24, 26). It was necessary to immunoprecipitate GRK6 to eliminate an endog-

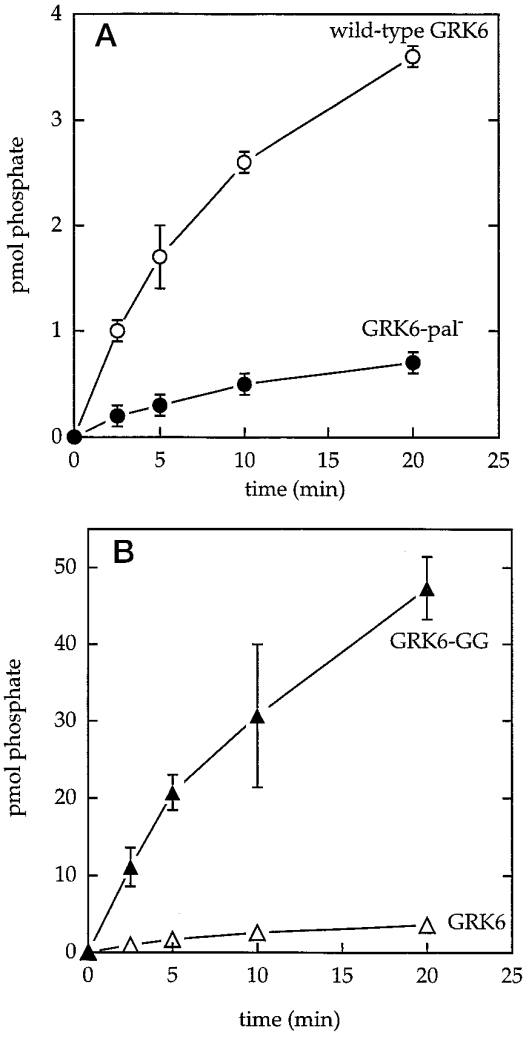

FIG. 2. **Time course of rhodopsin phosphorylation by wild-type and mutant GRK6.** Rhodopsin (6.8 μM) was phosphorylated with 2.5 ng of partially purified wild-type GRK6 and GRK6-pal$^-$ (*A*) or GRK6-GG (*B*) in buffer containing 200 μM [γ-^{32}P]ATP (1 cpm/fmol), 4.6 mM $MgCl_2$, 20 mM HEPES, pH 7.5, and 2 mM EDTA in room light. The reactions were incubated at 30 °C for the times indicated and were terminated by the addition of SDS sample buffer. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed. ^{32}P incorporation was determined by excising and counting the receptor bands. The data are presented as the mean ± S.E. of three to four experiments.

TABLE I
Kinetic parameters for wild-type and mutant GRK6

Kinetic parameters of the different GRK6 proteins for phosphorylating rhodopsin were determined by incubating 0.1–20 μM rhodopsin and 1.3 ng of GRK6 for 5 min at 30 °C as described under "Experimental Procedures." All values are presented as the mean ± S.E. from three to four independent experiments.

Kinase	K_m	V_{max}
	μM	*nmol P_i/min/mg*
GRK6	2.6 ± 0.4	231 ± 20
GRK6-pal$^-$	12.0 ± 0.5	110 ± 2
GRK6-GG	1.2 ± 0.1	3244 ± 209

enous phosvitin kinase activity present in COS-1 cells that was not removed during partial purification of GRK6 (data not shown). When wild-type GRK6 and GRK6-pal$^-$ immunoprecipitates were assayed using phosvitin as the substrate, comparable levels of phosphorylation were observed (data not shown). This is reflected in the similarity of the V_{max} values for

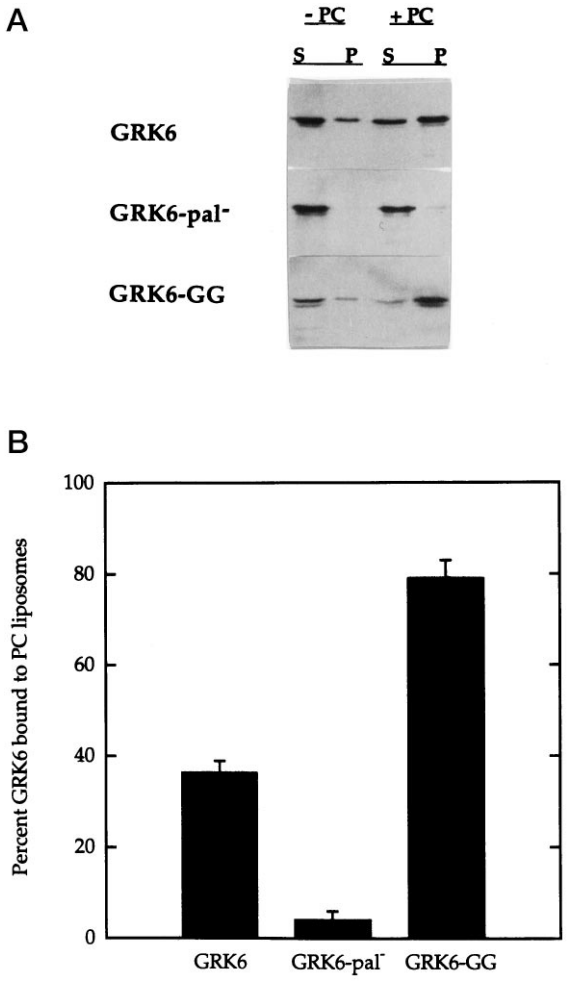

FIG. 3. **Binding of wild-type and mutant GRK6 to phosphatidylcholine vesicles.** ~15 ng of each partially purified GRK6 were incubated with or without 1.7 mg/ml sonicated soybean PC at 30 °C for 5 min. Samples were centrifuged at 100,000 × *g* for 10 min, electrophoresed on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, immunoblotted, and the percent GRK6 bound to PC vesicles quantitated as described under "Experimental Procedures." All values are expressed as the percent GRK6 bound to PC minus the percent GRK6 detected in the particulate fraction when incubated without PC. *Panel A*, Western blot analysis of one representative experiment; *S*, supernatant fraction, *P*, pellet fraction. *Panel B*, quantitation of GRK6 binding to PC vesicles. Data are presented as the mean ± S.E. of three experiments.

wild-type GRK6 (1.1 ± 0.4 nmol of P_i/min/mg) and GRK6-pal$^-$ (1.1 ± 0.3 nmol of P_i/min/mg) for phosvitin phosphorylation. Unfortunately, similar experiments with GRK6-GG could not be performed due to the inability of the antibody used in these studies to immunoprecipitate geranylgeranylated GRK6 (data not shown). These results demonstrate that mutation of cysteine residues 561, 562, and 565 does not impair GRK6 catalytic activity and that the observed differences in rhodopsin phosphorylation between wild-type and GRK6-pal$^-$ are most likely due to the palmitoylation state of GRK6.

Binding of GRK6 Proteins to Phosphatidylcholine Vesicles—To directly assess GRK6 binding to phospholipids *in vitro* we performed experiments in which the different GRK6 proteins were incubated in the presence or absence of sonicated PC vesicles at 30 °C. The samples were then centrifuged and the percent GRK6 bound to PC vesicles was determined by Western blotting (Fig. 3). Whereas wild-type GRK6 bound significantly to PC vesicles (36 ± 3%), GRK6-pal$^-$ did not bind (4 ± 2%), further supporting a role for palmitoylation in membrane binding of GRK6. In contrast, a higher level of GRK6-GG was

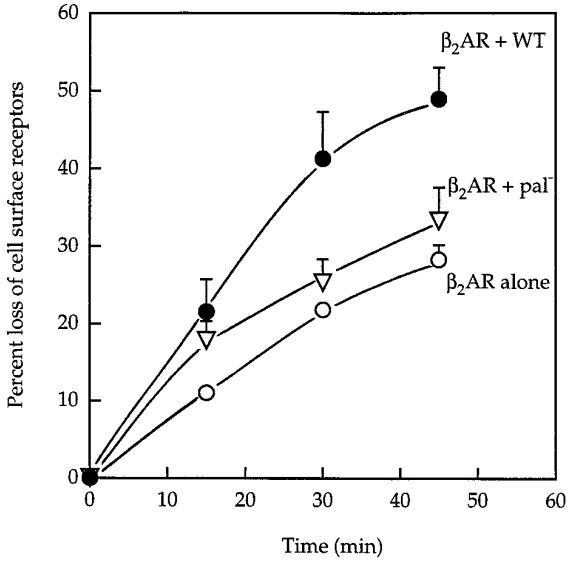

FIG. 4. **Isoproterenol-induced sequestration of β_2AR in COS-1 cells coexpressing wild-type and mutant GRK6.** COS-1 cells transfected with 8 μg of pBC-β_2AR with or without 8 μg of pBC-GRK6 or pBC-GRK6-pal$^-$ were harvested 48 h posttransfection and incubated for 0–45 min at 37 °C in the presence or absence of 10 μM (−)-isoproterenol. The cells were washed and then incubated with ^{3}H-CGP12177 to assess cell surface β_2ARs as described under "Experimental Procedures." The data are presented as the mean ± S.E. of four experiments.

bound to PC vesicles (79 ± 4%) compared with wild-type GRK6. This may be attributable to the more lipophilic properties of the geranylgeranyl isoprenoid *versus* palmitate. Alternatively, the differences in phospholipid binding between GRK6 and GRK6-GG may reflect the extent of posttranslational modification of the respective proteins expressed in COS-1 cells (*i.e.* wild-type GRK6 may not be completely palmitoylated).

Receptor Sequestration Assays—Recent studies have demonstrated an important role of GRKs in promoting agonist-induced sequestration of the β_2AR (30, 31). Thus, to assess the activity of wild-type and mutant GRK6 in intact cells, we transfected COS-1 cells with the β_2AR alone or together with wild-type GRK6 or GRK6-pal$^-$ and then measured agonist-induced sequestration. Isoproterenol-induced β_2AR sequestration was increased ~2-fold compared with control cells (Fig. 4). In contrast, GRK6-pal$^-$ was able to promote only a slight increase in receptor internalization *versus* control cells. This disparate pattern in receptor internalization was not due to lower expression of GRK6-pal$^-$. In fact, Western blot analysis revealed ~3-fold higher expression of GRK6-pal$^-$ compared with wild-type GRK6 (data not shown). Cells coexpressing receptor and GRK6-GG exhibited comparable levels of receptor internalization with those expressing wild-type GRK6 (data not shown) indicating that restoration of GRK6-pal$^-$ function is achieved when a CAAX motif is added to the C terminus of the mutant kinase. Taken together, these data strongly suggest that palmitoylation of GRK6 is necessary for its association to phospholipid membranes and, as such, provides a crucial regulatory component for kinase activity toward receptor substrates.

DISCUSSION

G protein-coupled receptor kinases utilize a number of different mechanisms to associate with cell membranes and phosphorylate their receptor substrates. Studies of rhodopsin kinase indicate that it undergoes farnesylation facilitating kinase translocation to the discal membrane of rod outer segments (20). A farnesylation-deficient mutant of rhodopsin kinase was unable to associate with the membrane and exhibited significantly reduced activity compared with the wild-type kinase (12).

βARK contains a region within the C terminus that is required for binding to membrane-associated G protein $\beta\gamma$ subunits (13). This region overlaps with the recently described PH domain that is found in a number of signal-transducing molecules including βARK (21). βARK phosphorylation of the β_2AR, m2 muscarinic acetylcholine receptor, and rhodopsin is significantly enhanced in the presence of $G_{\beta\gamma}$ (13, 32, 33). The N terminus of the βARK PH domain has been shown to facilitate βARK binding to phospholipid vesicles containing either phosphatidylinositol 4,5-diphosphate (PIP_2) or phosphatidylserine (14). In the absence of $G_{\beta\gamma}$, PIP_2 inhibited βARK activity whereas phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, and phosphatidylglycerol activated βARK ~2-fold. Purified $G_{\beta\gamma}$ subunits were able to reverse PIP_2 inhibition of βARK. Pitcher *et al.* (22) observed that both PIP_2 and $G_{\beta\gamma}$ were required for phospholipid binding and activation of βARK . A fusion protein containing the βARK PH domain blocked $G_{\beta\gamma}$/PIP_2-dependent activation of βARK. In contrast, a mutant fusion protein in which an invariant tryptophan residue present in all PH domains was mutated to an alanine showed no inhibitory activity. βARK2, which is expressed in olfactory epithelium, is involved in odorant receptor regulation and, like βARK, undergoes cytosol-to-membrane translocation and binds to $G_{\beta\gamma}$ subunits (16). In olfactory cilia, addition of a mixture of odorants results in translocation of βARK2 from the soluble to the membrane fraction (16). Taken together, these data suggest that although βARK and βARK2 are not directly acylated, these kinases may utilize $G_{\beta\gamma}$ subunits and phospholipids to associate with the plasma membrane.

The highly basic C terminus of GRK5 is important in membrane binding of the kinase, presumably through electrostatic interactions with polar fatty acid head groups (17, 23). Studies addressing the effects of membrane lipids on GRK5 activity demonstrated that phosphorylation of rhodopsin and the β_2AR is increased substantially in the presence of several phospholipids. Furthermore, GRK5 autophosphorylation was also enhanced under these conditions with autophosphorylated GRK5 having increased activity toward receptor substrates relative to the nonautophosphorylated kinase (17).

A number of signal transducing proteins, such as $G_{\alpha s}$ and the β_2AR, are reversibly palmitoylated (34, 35). Importantly, palmitoylation appears to modulate the function of these proteins. Activation of the β_2AR enhances the palmitate turnover rate of $G_{\alpha s}$ and may result in its membrane-to-cytosol translocation. This suggests that regulation of $G_{\alpha s}$ function may be due, in part, to a reversible cycle of palmitoylation-depalmitoylation that affects its cellular localization. That palmitoylation might be responsible for $G_{\alpha s}$ association to the plasma membrane is supported by the observation that a constitutively active mutant, α_s-R201C, exhibits accelerated palmitate turnover compared with wild-type $G_{\alpha s}$ and is found predominantly in the cytosol of cells stably expressing the mutant protein (34). Furthermore, the palmitate turnover rate of another mutant (α_s-G226A) that is refractory to β-agonist stimulation, is elevated only slightly in the presence of agonist compared with wild-type α_s and is not released from the particulate fraction (34). The β_2AR contains a palmitoylation site at Cys^{341} that is thought to form a putative fourth intracellular loop (36). Mutation of Cys^{341} to a glycine residue increases the basal level of receptor phosphorylation and decreases its ability to functionally interact with G_s. More detailed analysis of a mutant defective in palmitoylation (β_2AR-C341G) revealed that palmitoylation of the receptor regulates the accessibility of a protein

kinase A phosphorylation site located in the β_2AR C terminus and, thus, may control receptor responsiveness (36).

Recent studies addressing posttranslational modifications of GRKs demonstrate that GRK6 and the four splice variants of GRK4 are palmitoylated (18, 19). The overall architecture of GRKs is characterized by a centrally localized catalytic domain of high sequence homology flanked by a divergent N-terminal and variable-length C-terminal domains (37). The N terminus is thought to be important for GRK recognition of the activated receptor, whereas the C terminus appears to facilitate GRK binding to the cell membrane (37). Despite the strong functional evidence supporting the involvement of the C terminus in GRK localization to the membrane and in the enhancement of rhodopsin kinase, βARK, βARK2, and GRK5 activity, it was not known if palmitoylation regulates GRK4 and GRK6 in a similar manner. Our aim was to address this issue by generating a palmitoylation-deficient mutant of GRK6 and determining its activity toward rhodopsin and its ability to bind phospholipids *in vitro* as well as assess its activity in intact cells. The data presented in this study provide evidence that the palmitoylation state of GRK6 greatly influences its activity toward activated receptor substrates. Moreover, we postulate that the four GRK4 isoforms are similarly regulated since they contain the same cysteine cluster found in GRK6 and have been shown to undergo palmitoylation (19). However, the existence of four splice variants of GRK4 may indicate additional as yet ill-defined mechanisms that determine distinct regulatory features of these proteins.

Several lines of evidence suggest that nonpalmitoylated GRK6 is catalytically active but cannot readily associate with the cell membrane: 1) GRK6 and GRK6-pal$^-$ exhibit comparable activity toward the nonreceptor substrate phosvitin; 2) the V_{max} values for rhodopsin of GRK6-pal$^-$ and wild-type GRK6 are comparable (Table I); 3) wild-type GRK6 more readily binds to PC vesicles compared with GRK6-pal$^-$; and 4) addition of a C_{20} isoprenoid group to GRK6-pal$^-$ augments the ability of the kinase to phosphorylate rhodopsin and bind to PC vesicles *in vitro* (Figs. 2*B* and 3).

Experiments designed to assess β_2AR sequestration demonstrated that GRK6-pal$^-$ exhibits a significantly reduced ability to induce agonist-specific receptor internalization compared with wild-type GRK6. Furthermore, this defect is abolished in GRK6-pal$^-$ that contains a CAAX motif (data not shown). The ability of GRK6-pal$^-$ to promote a small but reproducible increase in receptor internalization suggests that the mutant kinase, in the absence of palmitoylation, still retains some function *in vivo*. This result is consistent with the observation that GRK6-pal$^-$ is able to phosphorylate rhodopsin, albeit with significantly lower activity compared with wild-type kinase. Taken together, these data provide the first evidence that kinase palmitoylation is crucial to its function *in vivo*.

We propose a model for GRK6 function in which palmitoylation facilitates kinase association with the cell membrane and allows for phosphorylation of activated receptor substrates. Since palmitoylation has been shown to be a highly dynamic and reversible process it is reasonable to postulate that, similar to $G_{\alpha s}$, a cycle of GRK6 palmitoylation-depalmitoylation is critical in mediating both agonist-specific receptor phosphorylation (leading to arrestin binding and eventual receptor sequestration) and dissociation of the kinase from the cell membrane and, hence, the receptor. Similar to the observation that agonist stimulation affects the incorporation of palmitate for $G_{\alpha s}$ is the possibility that receptor activation may alter GRK6 palmitate turnover, although this awaits formal proof. One could envisage, therefore, a tightly regulated system in which the activities of several different signal transduction components are reversibly controlled through this type of lipid modification.

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