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The G Protein-coupled Receptor Kinase 2 Is a Microtubule-associated Protein Kinase That Phosphorylates Tubulin*

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The G protein-coupled receptor kinase 2 (GRK2) is a serine/threonine kinase that phosphorylates and desensitizes agonist-occupied G protein-coupled receptors (GPCRs). Here we demonstrate that GRK2 is a microtubule-associated protein and identify tubulin as a novel GRK2 substrate. GRK2 is associated with microtubules purified from bovine brain, forms a complex with tubulin in cell extracts, and colocalizes with tubulin in living cells. Furthermore, an endogenous tubulin kinase activity that copurifies with microtubules has properties similar to GRK2 and is inhibited by anti-GRK2 monoclonal antibodies. Indeed, GRK2 phosphorylates tubulin *in vitro* with kinetic parameters very similar to those for phosphorylation of the agonist-occupied β_2-adrenergic receptor, suggesting a functionally relevant role for this phosphorylation event. In a cellular environment, agonist occupancy of GPCRs, which leads to recruitment of GRK2 to the plasma membrane and its subsequent activation, promotes GRK2-tubulin complex formation and tubulin phosphorylation. These findings suggest a novel role for GRK2 as a GPCR signal transducer mediating the effects of GPCR activation on the cytoskeleton.

Agonist occupancy of G protein-coupled receptors (GPCRs)[1] facilitates the exchange of bound GDP for GTP on heterotrimeric G proteins. The activated GTP bound G protein then dissociates into its constituent α- and $\beta\gamma$-subunits, both of which can activate a variety of different effector systems. The G protein-coupled receptor kinases (GRKs), a family of serine/threonine kinases, play an important role in regulating this signal transduction process (reviewed in Refs. 1–3). GRKs specifically phosphorylate agonist-occupied GPCRs, which are the only known substrates for these enzymes.

GRK-mediated phosphorylation of agonist-activated GPCRs promotes the high affinity binding of cytosolic arrestin proteins (β-arrestins) to the receptors (4, 5). β-Arrestin binding has two functional consequences. First, the binding of β-arrestin sterically inhibits coupling of the receptor to its respective G protein (4, 5). GRK-mediated receptor phosphorylation and β-arrestin binding thus lead to diminished receptor signaling, *i.e.* receptor desensitization (6). Second, β-arrestin binding initiates the clathrin-mediated endocytosis (sequestration) of activated receptors (7). GRK-mediated phosphorylation of activated GPCRs thus plays a critical role in regulating both the activity and number of plasma membrane receptors.

GRK2 is predominantly a cytosolic enzyme that becomes membrane-localized following GPCR activation (8, 9). The compartmentalization of GRK2 at the plasma membrane requires that its carboxyl-terminal pleckstrin homology domain binds both phosphatidylinositol 4,5-bisphosphate and the $\beta\gamma$-subunits of heterotrimeric G proteins ($G_{\beta\gamma}$) (10, 11). Since the membrane association of GRK2 requires free $G_{\beta\gamma}$ and the release of $G_{\beta\gamma}$ from the α-subunit is catalyzed by receptor activation, the membrane association of GRK2 is agonist-dependent. Thus GRK2 activity is regulated by several interdependent mechanisms. Agonist occupancy of the receptor and the targeting of GRK2 to different cellular compartments by $G_{\beta\gamma}$ regulate the rate of receptor phosphorylation by increasing the local GRK2 concentration. Additionally, allosteric activation of GRK2 occurs when it is complexed with $G_{\beta\gamma}$ and an activated receptor substrate (12, 13). This was demonstrated *in vitro* by measuring a potentiation of GRK-mediated phosphorylation of a peptide substrate in the presence of activated GPCR and $G_{\beta\gamma}$ (13). Thus, in addition to serving as GRK2 substrates, agonist-occupied GPCRs bind to and directly activate membrane-associated GRK2.

The activation of membrane-associated GRK2 by agonist-occupied GPCRs suggests, potentially, the existence of a signaling pathway in which GRK2 is the effector. To date, however, no substrates for these enzymes other than the receptors themselves have been found. Accordingly, we sought to identify GRK2-binding proteins and potential substrates by performing overlay assays and by examining the intracellular distribution of GRK2 using fluorescence and immunoelectron microscopy. Nitrocellulose overlay assays, in which protein extracts immobilized on nitrocellulose are incubated with a protein probe, have been successfully used to identify a number of proteins that interact with the regulatory subunits of protein kinase A, termed A kinase anchoring proteins (reviewed in Ref. 14). Protein kinase C-binding proteins (receptors for activated protein kinase C) (reviewed in Ref. 15) and protein kinase C substrates (16–18) have also been identified using similar procedures. In this study, we identify tubulin as a GRK2-binding protein and a novel GRK2 substrate. The potential implications

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; β-AR, β_2-adrenergic receptor; HEK, human embryonic kidney; PBS, phosphate-buffered saline; $G\beta\gamma$, the $\beta\gamma$ subunits of heterotrimeric G proteins; GFP, green fluorescent protein; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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of GRK-mediated tubulin phosphorylation on GPCR function are discussed.

EXPERIMENTAL PROCEDURES

Materials

Propranolol and isoproterenol were from Sigma or RBI. Anti-mouse and anti-rabbit antibodies were obtained from Sigma and Molecular Probes, Inc. Mouse monoclonal antibodies against the 12CA5 (hemagglutinin) epitope were purchased from Boehringer Mannheim, and monoclonal M2 anti-Flag® antibody was purchased from Kodak IBI. Cell culture media were purchased from Medtech, and fetal bovine serum was purchased from Atlanta Biologicals. Physiological buffers were from Life Technologies, Inc. Restriction enzymes were obtained from Promega or New England Biolabs, T4 DNA ligase from Promega, and Hot Tub DNA polymerase from Amersham Pharmacia Biotech. Plasmids containing variants of green fluorescent protein were purchased from CLONTECH.

Plasmid Construction

GRK2-Flag—The Flag peptide sequence (DYKDDDDK) was inserted by site-directed mutagenesis before the C-terminal leucine residue of the GRK2 backbone residing in the vector pcDNA1/Amp. A cDNA fragment coding for the insert was ligated between the XhoI restriction site of GRK2 and the *Sal*I site of pcDNA1/Amp and verified by sequencing.

GRK2-Flag-GFP—A mutant GFP (pS65T-GFP) with a red shifted excitation spectrum and enhanced fluorescence compared with wild type GFP was attached to the C terminus of the Flag® epitope tagged GRK2 (19). The (TAA) stop codon following the C-terminal leucine was replaced using site-directed mutagenesis (20) with an in frame *Bam*HI restriction site. The proximal *Hin*dII/*Xho*I fragment was ligated with the *Xho*I/*Bam*HI fragment into (pS65T-GFP) between the *Hin*dIII/*Bam*HI polylinker restriction sites.

Fractionation of Bovine Tissue Extracts

To survey for the presence of GRK2-binding proteins, various bovine tissues (frozen in liquid nitrogen), were thawed and homogenized (5 ml/g, wet weight) in buffer A (20 mM Tris, pH 7.2, containing 0.25 M sucrose, 5 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 10 mM benzamidine-HCl). Tissue samples were homogenized using a Polytron homogenizer, and nuclei were pelleted by centrifugation at 700 × *g* for 15 min. The supernatant, termed crude homogenate, was further fractionated into particulate and soluble fractions by centrifugation at 150,000 × *g* for 1 h. The resulting particulate fractions were resuspended in buffer A (5 ml/g of tissue in the original homogenate). All operations were performed at 4 °C. Protein concentrations were determined with Bradford reagent (Bio-Rad) using bovine serum albumin as a standard.

Overlay Method for Detection of GRK2-binding Proteins

GRK2-binding proteins were identified using a modification of a procedure initially described by Leiser *et al.* (21). Proteins in samples to be probed were separated by SDS-polyacrylamide gel electrophoresis (22) and electrophoretically transferred to nitrocellulose membranes. The nitrocellulose filters were incubated in blotto (10 mM potassium phosphate buffer, pH 7.4, 0.15 M NaCl, 5% (w/v) nonfat dry milk, and 0.02% NaN_3) for 1 h at 4 °C and subsequently washed three times with binding buffer (100 mM Tris, pH 7.4, 50 mM NaCl). GRK2-binding proteins were detected by incubating the nitrocellulose filters with purified autophosphorylated GRK2. GRK2 (3 μM) purified from baculovirus-infected Sf9 cells, described by Kim *et al.* (23), was autophosphorylated by incubation in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2.0 mM EDTA, 1 mM dithiothreitol containing 60 μM ATP (~6000 cpm/pmol) at 30 °C for 30 min. Prior to incubation with the nitrocellulose filters, the GRK2 was desalted over G25 columns (1 ml) to remove excess [γ-^{32}P]ATP. The ^{32}P-labeled GRK2 (0.2 μM) was incubated with the nitrocellulose filters in binding buffer for 1 h at 4 °C. Blots were washed extensively with binding buffer to reduce nonspecific binding and were subsequently exposed to x-ray film.

Purification of Taxol-precipitated Microtubules and Tubulin

Purified microtubules containing microtubule-associated proteins were prepared from homogenates of bovine brain using the antimitotic drug taxol as described by Vallee (24).

Purified tubulin was prepared from extracts of freshly isolated bovine brain as described by Simon *et al.* (25). Briefly, brain was homogenized at a ratio of 0.5 ml of buffer/g of tissue in 100 mM Pipes, pH 6.9, containing 2 mM EGTA and 1 mM $MgSO_4$ (PEM buffer), that also contained 1 mM ATP and protease inhibitors. The homogenate was centrifuged at 100,000 × *g* for 1 h at 4 °C, and the supernatant was diluted 1:1 with PEM containing 60% glycerol and 0.2 mM GTP (PEMG buffer). After a 45-min incubation to polymerize tubulin, microtubules were collected by centrifugation at 100,000 × *g* for 45 min at 29 °C. The microtubule pellet was processed through a second depolymerization/polymerization step by cycling between 4 °C and 37 °C. The two-cycle purified tubulin was subsequently purified to >99% homogeneity using phosphocellulose chromatography as described by Voter and Erickson (26). Purified tubulin was stored in aliquots at −80 °C until use.

Western Blots

Western blots were performed by standard procedures using monoclonal antibodies against GRK2 (27) and polyclonal or monoclonal antibodies directed against β-tubulin (Sigma). Enhanced chemiluminescence detection of antigens (DuPont) was achieved with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech).

Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells were maintained in minimal essential medium or Dulbecco's modified Eagle's medium with 10% fetal bovine serum and penicillin/streptomycin in a 5% CO_2 incubator at 37 °C. Cells were transfected with 2.0–5.0 μg of plasmid containing GRK2-Flag-GFP cDNA using coprecipitation with calcium phosphate (28). Cells were maintained in 100-mm dishes or transferred to 22-mm square, ethanol-sterilized coverslips in six-well plates as necessary. Cell lines permanently expressing GRK2-Flag-GFP or the GRK2-Flag construct were made using G418 (Geneticin) selection (0.5 mg/ml) of calcium phosphate-transfected HEK-293 cells. Plasmids encoding bovine GRK2 (28) and the human M2 Flag-tagged β_2-adrenergic receptor in pcDNAs (28) were also used in this study.

Immunoprecipitation of GRK2 and Tubulin

Serum-starved HEK-293 cells overexpressing GRK2 or GRK2 and β_2-adrenergic receptor were treated with agonists as described in the figure legends. Medium was subsequently removed, and cell monolayers were washed twice with ice-cold phosphate-buffered saline (PBS). Cells were subsequently lysed by scraping into 1% CHAPS-HEDN buffer (HEDN contained 10 mM Hepes, pH 7.2, 1 mM EDTA, 1 mM dithiothreitol, and 100 mM NaCl), 1 ml of buffer per 150-mm plate of 80% confluent cells. Lysates were cleared by centrifugation at 15,000 × *g* for 15 min at 4 °C, and the supernatants incubated with 15 μg of immunoprecipitating antibody. A monoclonal anti-β-tubulin antibody (Sigma) (see Figs. 2 and 11) or a monoclonal anti-GRK2 antibody (27) (see Fig. 10) was used. Incubations were performed at 4 °C for 1 h in the presence of 50 μl of a 50% slurry of protein A/G-Sepharose (Calbiochem). Following this incubation period, protein A/G-Sepharose-bound immune complexes were recovered by centrifugation and washed three times in CHAPS-HEDN. Proteins were removed from the Sepharose beads with SDS-polyacrylamide gel electrophoresis sample buffer (8% SDS, 25 mM Tris, pH 6.5, 10% glycerol, 5% mercaptoethanol, 0.003% bromphenol blue), resolved by electrophoresis on 12% acrylamide gels, and subjected to Western blot analysis.

Phosphorylation Reactions

Phosphorylation of Tubulin by the Microtubule-associated Tubulin Kinase—Taxol-precipitated microtubules (200 nM) were incubated in a volume of 25 μl in 20 mM Tris, pH 7.5, 2.0 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol containing 60 μM [γ-^{32}P]ATP (~6000 cpm/pmol) (buffer B). Incubations were performed at 30 °C for the times indicated in the figure legends. Reactions were stopped by the addition of an equal volume of SDS sample loading buffer and electrophoresed on 10% SDS-polyacrylamide gels. The dried gels were subjected to autoradiography and PhosphorImager (Molecular Dynamics) analysis to determine the number of pmol of phosphate transferred to tubulin. Incubation with protein kinase A inhibitor (10 μg/ml), staurosporine (10 nM), heparin (5 μM), GTP (10 mM), and monoclonal antibodies (10 μg) was used to elucidate the biochemical characteristics of the microtubule associated tubulin kinase.

GRK2-mediated Phosphorylation of Tubulin—Phosphorylation reactions were performed essentially as described above with two exceptions. First, tubulin purified by phosphocellulose chromatography and devoid of endogenous kinase activity was used as a substrate. Second, purified recombinant GRK2 (50 nM) (23) was included in the phospho-

rylation reactions. Tubulin concentrations ranging between 0.03 and 0.9 μM were incubated for 10 min at 30 °C to determine the kinetic parameters for GRK2-mediated tubulin phosphorylation.

GRK2-mediated Phosphorylation of Receptor Substrates—Purified rod outer segment membranes (29) or purified reconstituted β-AR (10, 30) were incubated in buffer B at 30 °C with 50 nM GRK2. Phosphorylation reactions were incubated and analyzed as described under "Phosphorylation of Tubulin by the Microtubule-associated Tubulin Kinase." β-AR concentrations ranging between 0.03 and 0.9 μM were incubated at 30 °C for 10 min to determine the kinetic parameters of GRK2-mediated β-AR phosphorylation. A rhodopsin concentration of ~30 μM was used in assays utilizing this substrate.

GRK-mediated phosphorylation of a soluble synthetic peptide substrate—A stock solution of the purified peptide (RRREEEEESAAA) was prepared, and the pH was adjusted to 7.2 by the addition of Tris base. GRK-mediated peptide phosphorylation was determined by incubating peptide (10 μM to 1 mM) and GRK2 (50 nM) in 20 mM Tris-HCl, pH 7.2, 2 mM EDTA, 7.5 mM $MgCl_2$, and 60 μM [γ-^{32}P]ATP (~2000 cpm/pmol). The final reaction volume was 25 μl, and incubations were performed at 30 °C for 15 min. Phosphorylation reactions were linear over this time period. Reactions were stopped by spotting onto P-81 phosphocellulose paper (2 × 2-cm squares). Free [γ-^{32}P]ATP was subsequently removed by washing in 75 mM phosphoric acid as described previously (31). GRK2-mediated peptide phosphorylation was determined by subtracting the counts incorporated in the absence of peptide from the counts incorporated in the presence of this substrate.

Phosphorylation of Cellular Tubulin

HEK-293 cells transiently overexpressing GRK2 and β-AR were starved in phosphate-free Dulbecco's modified Eagle's medium (Life Technologies, Inc.) for 2 h. These cells were subsequently incubated in the same medium containing [^{32}P]orthophosphate (0.2 mCi/ml) for 2 h to label intracellular pools of ATP. Cells were treated with the β-adrenergic agonist isoproterenol (10 μM for 10 min), washed three times with ice-cold PBS, and harvested in 20 mM Tris, pH 7.4, 2 mM EDTA containing protease inhibitors. Following a low speed spin to remove nuclei (600 × g for 15 min), membranes were prepared by spinning the clarified cellular homogenate at 150,000 × g for 20 min. Tubulin was subsequently immunoprecipitated from these cells as described under "Immunoprecipitation of GRK2 and tubulin."

Immunofluorescence, Interference-Contrast, and Video Microscopy

GRK2-Flag-GFP or GRK2-Flag-expressing HEK-293 cells transfected as described were plated onto ethanol-sterilized glass coverslips in growth medium at least 24 h prior to observation. Coverslips were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Antibody labeling or washing of fixed cells was performed at room temperature in a solution of PBS containing 0.008% saponin (w/v) and 1% bovine serum albumin at pH 7.2. A primary rabbit, anti-tubulin antibody originally raised against sea urchin tubulin, a gift of Dr. K. Fujiwara, was kindly provided by Prof. Harold Erickson (Duke University) and was used at a 1:1000 dilution. The mouse monoclonal M2 anti-Flag® epitope antibody was used to localize GRK2-Flag. All antibody incubations were performed at room temperature for 40–60 min with three or four washes following each incubation. Either fluorescein or Texas Red-conjugated secondary antibody (anti-mouse or anti-rabbit) was used as required at 1:250 dilutions. Coverslips were inverted, mounted on glass slides over a drop of PBS, and sealed with clear nail polish prior to viewing. Samples were observed with a Leica model DM50 epifluorescence microscope with one port connected to an Optronics VI-470 CCD video camera system with 768 × 494 active pixels set in manual gain mode. GRK2-Flag-GFP fluorescence and fluorescein fluorescence were visualized using a fluorescein (GFP) excitation and emission filter cube, whereas Texas Red was observed using a broad band excitation rhodamine cube. The electronic cell images obtained from the camera were printed using a Sony model UP-5600 MD color video printer with a UPK-5502SC digital interface board, and imported into Adobe Photoshop (2.5) using the accompanying Sony import module.

Sequestration

Flow cytometry analysis was performed as follows. GRK2, GRK2-Flag, or GRK2-Flag-GFP was coexpressed in HEK-293 cells with the 12CA5 epitope-tagged Y326A mutant β-AR (32). Cells were grown in six-well Falcon dishes at a density of 250,000–400,000 cells/well with equal seeding per well. Following aspiration and washing of each well with serum-free medium, serum-free media with or without isoproterenol was added at 37 °C for 30 min. The incubations were stopped by aspiration of medium and the addition of ice-cold PBS to each well. Following washing in PBS, the cells were incubated for 30 min with a 1:400 dilution of anti-12CA5 antibody in Dulbecco's modified Eagle's medium at 4 °C, washed three times in cold PBS, incubated with a 1:250 dilution of goat anti-mouse R-phycoerythrin-conjugated antibody, and then fixed and stored in 3% formaldehyde for flow cytometry. 50,000 cells were analyzed for each condition using 520-nm excitation.

Immunoelectron Microscopy of HEK-293 Cells for GRK2-Flag and Tubulin

Confluent 100-mm dishes of permanently transfected, GRK2-Flag-expressing HEK-293 cells or untransfected cells were fixed for 20 min with 4% paraformaldehyde/PBS, washed in PBS, and treated at room temperature for 60 min with a 0.008% saponin, 1% bovine serum albumin PBS solution containing a 1:500 dilution of M2 anti-Flag antibody or a 1:1000 dilution of rabbit anti-tubulin antibody. They were then washed three times with PBS to remove free antibody and prepared for electron microscopy as follows. Cells were pelleted, further fixed in paraformaldehyde in 200 mM Pipes, pH 7.0, coated with agar to hold them together, infiltrated with 2.1 M sucrose for cryoprotection, placed onto stubs, and then snap-frozen in liquid nitrogen. They were stored in a liquid nitrogen freezer until sectioned. Ultrathin cryosections were cut on a Reichert-Jung ultracut E, equipped with an FC4 cryochamber (Leica, Deerfield, IL). Sections were collected on Formvar and carbon-coated nickel grids, incubated on 5% fetal calf serum in PBS, and followed by 50 mM ammonium chloride in PBS. Grids not previously treated with anti-tubulin primary antibody were incubated over a 1:100 dilution of rabbit anti-tublulin antibody for 1 h at room temperature and washed. Grids were further labeled by incubation with goat anti-mouse and goat anti-rabbit IgG conjugated with either 5-nm or 10-nm colloidal gold (Aurion) at a 1:10 dilution. After thorough washing in PBS followed by washing in water, they were embedded in a 9:2 mixture of 2.1 M methyl cellulose and 2% aqueous uranyl acetate. Grids were viewed in a Philips EM300 electron microscope.

RESULTS AND DISCUSSION

Detection of GRK2-interacting Proteins by Protein Overlay—Crude extracts (C) derived from various bovine tissues, together with a soluble (S) and a particulate (P) fraction derived from this extract (Fig. 1*A*) were subjected to electrophoresis on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose. The nitrocellulose filters were subsequently incubated with a purified preparation of autophosphorylated ^{32}P-labeled GRK2. Following extensive washing, GRK2 retained on the filter was detected by autoradiography. As shown in Fig. 1*A*, very few GRK2-binding proteins were detected under these conditions. GRK2 was retained on the filter by proteins of 55-kDa present in the crude extracts and particulate fraction derived from bovine brain and retina. Additionally, a 42-kDa GRK2-binding protein was detected in the crude and particulate fraction derived from bovine heart. A similar pattern of GRK2 binding proteins was obtained when nitrocellulose filters were incubated with unphosphorylated GRK2, and bound GRK2 was detected immunologically (data not shown).

Partial purification of the 55-kDa GRK2-binding protein from bovine brain revealed that this protein is tightly associated with the particulate fraction and, as compared with other bovine tissues, is highly enriched in brain. The properties and molecular weight of this protein are consistent with those of tubulin. The identity of the 55-kDa GRK2 binding protein was confirmed as tubulin based on two findings: (i) partially purified preparations of the GRK2-binding protein cross-react with an anti-tubulin antibody, and (ii) purified tubulin migrates with a molecular weight identical to that of the GRK2-binding protein in brain extracts and binds GRK2 in the overlay assay (Fig. 1*B*).

GRK2 Is Associated with Tubulin in Intact Cells—Tubulin is an extremely abundant protein representing approximately 5% of the total protein content of brain. Furthermore, in the nitrocellulose overlay assay, native GRK2 binds to denatured, immobilized tubulin. In light of these observations, is the inter-

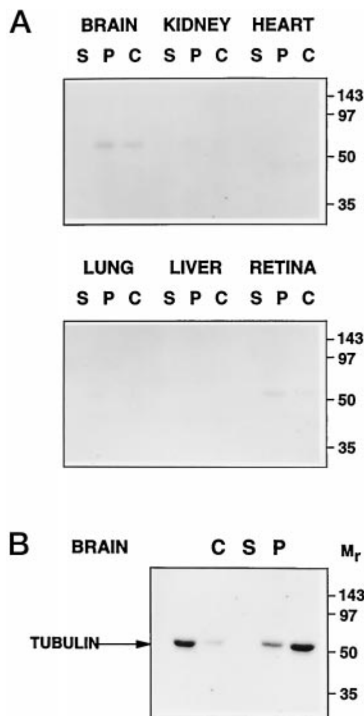

FIG. 1. **Detection of GRK2-binding proteins by nitrocellulose overlay assay.** *A* and *B*, nitrocellulose overlay assays were performed, as described under "Experimental Procedures," using ^{32}P-labeled GRK2 as a probe. Bound GRK2 was visualized following exposure of the nitrocellulose filters to film. Soluble (*S*), particulate (*P*), and crude extract (*C*) fractions from the indicated bovine tissues were probed for binding proteins. 15 μg of total protein was run in each lane. *B*, 15 μg of a purified microtubule preparation was run in *lanes 1* and *5*. The migration positions of molecular weight standards and purified tubulin, as determined by Ponceau S staining, are indicated. The blot shown is representative of at least three separate experiments.

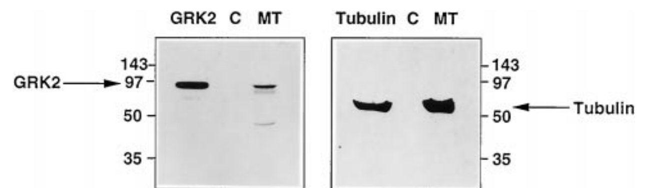

FIG. 2. **GRK2 copurifies with microtubules.** 25 μg of a crude bovine brain extract (*C*) or a purified microtubule preparation (*MT*) was subjected to Western blot analysis using a monoclonal anti-GRK2 (*left panel*) or anti-tubulin antibody (*right panel*). Purified GRK2 and tubulin were included on the blots as controls. The migration positions of molecular weight standards, GRK2, and tubulin are indicated. Similar results were obtained using three different preparations of taxol-precipitated microtubules.

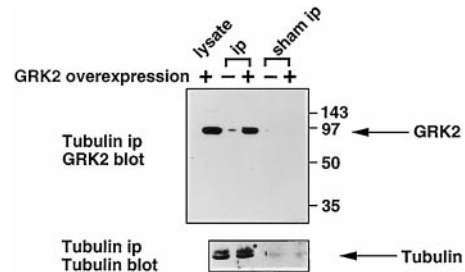

FIG. 3. **GRK2 coimmunoprecipitates with tubulin.** Tubulin from HEK-293 lysates (approximately 1 mg of protein) was immunoprecipitated using a monoclonal anti-β tubulin antibody (*ip*). Cells were transfected with GRK2 where indicated. Control immunoprecipitations (*sham ip*) in which the immunoprecipitating antibody was omitted were also included. Immunoprecipitates were subjected to Western blot analysis using an anti-GRK2 antibody (*upper panel*) or a polyclonal anti-tubulin antibody (*lower panel*). The migration positions of molecular weight standards and GRK2 are indicated. The Western blot shown is representative of three separate immunoprecipitations.

action between GRK2 and tubulin of physiological significance? That GRK2 binds to native tubulin and that this interaction occurs in intact cells is shown in Figs. 2, 3, 5, and 6.

Microtubule preparations purified from bovine brain extracts using the antimitotic drug taxol were probed immunologically for GRK2 and tubulin (Fig. 2). Taxol-induced precipitation of microtubules was performed under conditions of low ionic strength to ensure copurification of accompanying microtubule-associated proteins. Western blotting of equal amounts of a crude extract (*C*) and a taxol-precipitated microtubule preparation (*MT*) reveals a dramatic enrichment in both GRK2 and tubulin content following microtubule purification (Fig. 2), results that indicate a physical association between GRK2 and native tubulin.

The existence of a GRK2-tubulin complex can also be demonstrated by coimmunoprecipitation of these two proteins from lysates of HEK-293 cells (Fig. 3). As shown in Fig. 3, Western blot analysis of tubulin immunoprecipitates reveals the presence of GRK2. This represents a specific interaction between GRK2 and tubulin, as indicated by two observations. First, no GRK2 immunoreactivity is detected in sham immunoprecipitations, *i.e.* in the absence of immunoprecipitating tubulin antibody (compare *ip* and *sham ip* in Fig. 3). Second, increasing the cellular content of GRK2 by transient overexpression dramatically increases the amount of this enzyme specifically immunoprecipitated with tubulin (Fig. 3).

Thus, as assessed by two different experimental approaches, copurification and coimmunoprecipitation, GRK2 and tubulin form a complex under physiologically relevant conditions. One prediction from these observations is that GRK2 should colocalize with tubulin in an intact cell. To study the intracellular distribution of GRK2 stable HEK-293 cell lines were made permanently expressing GRK2 constructs that could be immunolocalized with monoclonal antibodies to an epitope tag (GRK2-Flag) or by the intrinsic fluorescence of the green fluorescent protein (GRK2-Flag-GFP). Characterization of wild type GRK2 and GRK2-Flag reveal these enzymes to have equivalent activities and identical patterns of G$\beta\gamma$ sensitivity when assayed against rhodopsin *in vitro* (Fig. 4*A*). GRK2-Flag-GFP also phosphorylated rod outer segment *in vitro* but displayed a somewhat reduced activity and G$\beta\gamma$ sensitivity as compared with GRK2 and GRK2-Flag (Fig. 4*A*). Notably, however, overexpression of all GRK2 constructs in HEK-293 cells enhanced sequestration of a sequestration impaired β-AR mutant (Y326A-β-AR) (Fig. 4*B*). Agonist-mediated phosphorylation of the Y326A-β-AR by GRK2 facilitates the internalization of the β-AR and indicates that the kinase interacts with the receptor in an agonist-dependent manner (32, 33). Overexpression of GRK2-Flag and GRK2-Flag-GFP in HEK-293 cells results in a rescue of the Y326A-β-AR sequestration, similar to that observed following wild type GRK2 expression (Fig. 4*B*). Wild type GRK2, GRK2-Flag, and GRK2-Flag-GFP would thus appear functionally equivalent when expressed in cells.

Fluorescence microscopy was used to examine the distribution of GRK2 and tubulin in intact HEK-293 cells. The inherent fluorescence of the GFP was used to visualize GRK2 and a polyclonal anti-tubulin antibody, coupled with a Texas Red-conjugated secondary antibody, to visualize tubulin. In interphase cells, filamentous cytoplasmic microtubules distributed throughout the HEK cell cytoplasm were observed (data not shown), and GRK2-Flag-GFP appeared to be homogeneously distributed throughout the cytoplasm. To distinguish between GRK2-Flag-GFP colocalized with tubulin and free GRK2-Flag-

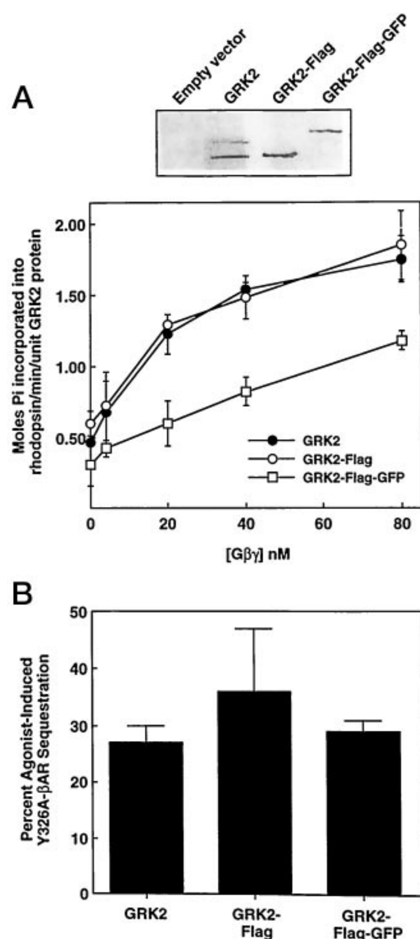

FIG. 4. **Expression and activity of GRK2, GRK2-Flag, and GRK2-Flag-GFP.** *A*, GRK2, GRK2-Flag, and GRK2-Flag-GFP phosphorylate rhodopsin in a Gβγ-dependent fashion. Lysates (5 μg of protein) from cells transfected with empty vector, GRK2, GRK2-Flag, or GRK2-Flag-GFP were assayed for their ability to phosphorylate rhodopsin in the presence of the indicated concentrations of Gβγ. The results are expressed as pmol of P_i incorporated into rhodopsin/min/arbitrary unit of GRK2 protein. Cells expressing empty vector were used to determine the extent of rhodopsin phosphorylation in the absence of GRK2 overexpression, a value that was subsequently subtracted from that obtained using lysates overexpressing GRK2. The amount of GRK2 protein present in the assay was determined by Western blot analysis. The results presented represent the mean ± S.E. of three separate experiments. A representative Western blot showing the levels of GRK2 expressed in the four cell lines used is also shown. *B*, the sequestration of Y326A-β-AR in HEK-293 cells is rescued to the same degree by overexpression of either GRK2-Flag or GRK2-Flag-GFP. Results are presented from triplicate experiments (mean ± S.E.).

GFP, we investigated cells in which tubulin becomes locally concentrated, such as in mitotic spindles or taxol-induced cytoplasmic aggregates (34, 35). GRK2-Flag-GFP localization to spindle structures was consistently seen (Fig. 5). Fig. 5, *A–C*, shows a mitotic cell in, respectively, interference contrast, tubulin fluorescence (red), and GRK2-Flag-GFP fluorescence (green). The pattern of tubulin over the mitotic spindle (*B*) is replicated by the distribution of GRK2-Flag-GFP fluorescence over the same region (*C*). Fig. 5, *D* and *E*, show another mitotic cell by tubulin fluorescence (*D*) and GRK2-Flag-GFP fluorescence (*E*). Fig. 5 also demonstrates colocalization of these two proteins in regions of postmitotic tubulin condensation near the edges of two daughter cells (*panel F*, tubulin fluorescence; *panel G*, GRK2 fluorescence). The appearance of tubulin fluorescence and absence of GRK2-Flag-GFP fluorescence in a non-transfected cell (*top right corner* of *panels F* and *G*) indicates that fluorescent cross-talk is not contributing to the GRK2

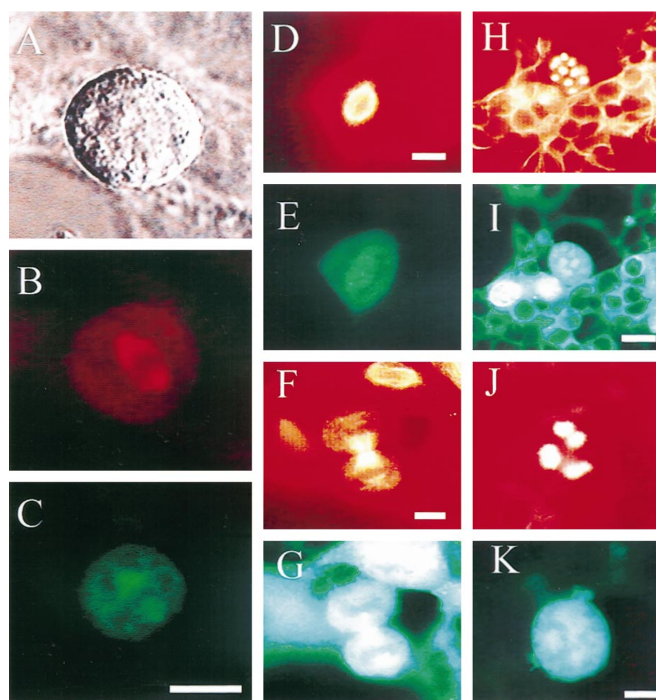

FIG. 5. **Association of GRK2-Flag-GFP and tubulin in HEK-293 cells.** *A–C*, serial images of a mitotic HEK-293 cell by interference contrast (*A*), tubulin fluorescence (*B*), and GRK2-Flag-GFP fluorescence (*C*). Note the colocalization of the tubulin with the GFP-conjugated GRK2 in the paired images *B* and *C*. Paired images of tubulin and GRK2-Flag-GFP fluorescence in mitotic cells (*D–G*) and cells treated with 10 nM taxol (*H–K*) for 1.5 h at 37 °C. GFP fluorescence from the GRK2-Flag-GFP conjugate is enhanced in regions containing large concentrations of tubulin. This can be observed in the mitotic spindle in the center of the cell stained for tubulin in D and imaged for GRK2-Flag-GFP fluorescence in *E* or in the two daughter cells stained for tubulin (*F*) or viewed by GRK2-Flag-GFP fluorescence (*G*). Taxol-produced cytoplasmic aggregates yield qualitatively similar results when viewed for tubulin (*H*, *J*) and GRK2-Flag-GFP (*I*, *K*). *Bars* correspond to 50 μm for *A–C*, 25 μm for *D–G* and *J–K*, and 62 μm for *H–I*).

signal. A permanently transfected cell line expressing GFP alone did not show the fluorescence enhancement over mitotic spindles observed with GRK2-Flag-GFP (data not shown). In HEK-293 cells treated with taxol, tubulin becomes redistributed into large cytoplasmic aggregates (Fig. 5, *H* and *J*). The colocalization of GRK2-Flag-GFP fluorescence with these tubulin aggregates is shown also in *panels I* and *K*. In cells overexpressing the GFP alone, the distribution of this protein does not qualitatively mimic that of tubulin in taxol-treated cells.

To investigate the association of GRK2 and tubulin more closely, we examined by immunoelectron microscopy the distribution of tubulin in HEK-293 cells that permanently express the GRK2-Flag construct. The results of the immunogold localization are shown in Fig. 6. The images demonstrate the absence of nonspecific staining in prefixed and saponin-treated cells not exposed to either primary antibody but then treated with immunogold (Fig. 6*A*). Fig. 6*B* shows 10-nm immunogold staining for tubulin where long stretches of tubulin labeling can be observed. In Fig. 6*C*, cytoplasmic GRK2, labeled with 10-nm immunogold, is scattered throughout the cytoplasmic compartment and is not found in the nucleus; in agreement with the immunofluorescence results. GRK2 also binds at mitochondrial surfaces, and, most interestingly, some GRK2 appears linearly distributed along filaments originating or terminating at mitochondrial membranes. To determine if these filaments contained tubulin, we simultaneously stained cells for both proteins (Fig. 6*D*). The larger 10-nm gold particles correspond to tubulin, and the smaller 5-nm ones correspond to

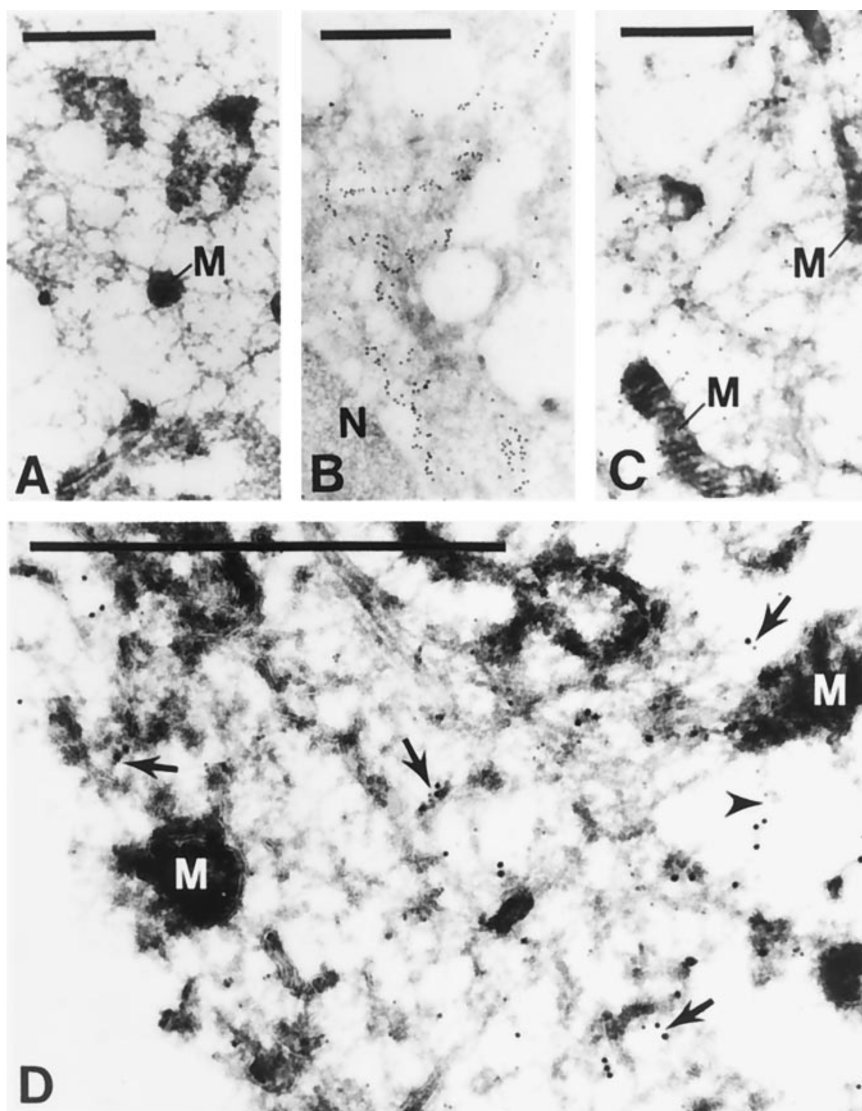

FIG. 6. **Immunoelectron micrographs of HEK-293 cells transfected with GRK2-Flag.** *A–C*, HEK cells were labeled as described under "Experimental Procedures." The cells were labeled with no primary antibody (*A*), rabbit anti-tubulin (*B*), and mouse anti-Flag antibody, respectively (*C*), followed by either immunogold-conjugated goat anti-rabbit antibody or immunogold-conjugated goat anti-mouse antibody. *A*, treated with both secondary antibodies and exhibits essentially no labeling. *B*, treated with anti-rabbit 10-nm immunogold. Linear distributions of tubulin can be observed throughout the image. In *C*, GRK2-Flag can be observed using anti-mouse 10-nm gold to follow linear patterns and is localized at the surface of mitochondria. *D*, this cell field was stained for both GRK2-Flag and tubulin. Tubulin is labeled by the larger 10-nm gold particles, whereas GRK2-Flag is labeled by 5-nm gold, with the *arrows* highlighting sites of colocalization. Note the distribution of GRK2-Flag and tubulin on the *right* (*arrowhead*) that follows a filament back to the mitochondrial surface. *Bars* correspond to 720 nm in *A–C* and 360 nm in *D*. *M*, mitochondria; *N*, nucleus.

GRK2. No reactivity was observed between the rabbit anti-tubulin and goat anti-mouse 5-nm immunogold, nor between the mouse monoclonal anti-Flag antibody and the 10-nm anti-rabbit immunogold (data not shown). Sites of GRK2 colocalization with tubulin are apparent (Fig. 6*D*, *arrows*), with a curved filament stained by both GRK2 and tubulin seen to terminate at a mitochondrial membrane (Fig. 6*D*, *arrowhead*). These results suggest that under these conditions a significant fraction of cellular GRK2 colocalizes with tubulin, results that are in agreement with the fluorescence data. The demonstration by two distinct localization techniques, as well as by biochemical approaches, of an intracellular association between GRK2 and tubulin, is strongly suggestive of a functionally relevant interaction between these two proteins.

Tubulin Kinase and GRK2 Have Similar Biochemical Properties—Microtubules consist of a core cylinder built from heterodimers of α- and β-tubulin monomers (36). As many as six different genes encode for α- and β-tubulin, a heterogeneity that is further increased by the postranslational modification of these proteins (37). One such post-translational modification is phosphorylation. Phosphorylation of tubulin on both α- and β-subunits has been reported, although to date only class IIIβ-tubulin from adult brain has been shown to be phosphorylated *in vivo* (38, 39). The identity of the serine/threonine kinase responsible for this phosphorylation event remains obscure. Ca^{2+}/calmodulin-dependent protein kinase (40), casein kinase I (41), and casein kinase II (42, 43) can phosphorylate tubulin *in vitro*, although it is currently unknown if these kinases phosphorylate tubulin *in vivo*. A tubulin kinase activity with biochemical properties similar to casein kinase II has, however, been reported to copurify with microtubules (44). Indeed, we find that the addition of Mg^{2+}/ATP to a taxol-precipitated microtubule preparation is sufficient to promote tubulin phosphorylation (Fig. 7). Since GRK2 is tightly associated with tubulin (Figs. 2, 3, 5, and 6), could GRK2 be a tubulin kinase, possibly even the main microtubule-associated tubulin kinase? To investigate this possibility, the biochemical characteristics of the microtubule-associated tubulin kinase were compared with those of purified GRK2. Protein kinase A inhibitor, staurosporine (a protein kinase C inhibitor), heparin (an inhibitor of casein kinase II (45) and members of the GRK family (46)), and GTP were used as potential inhibitors of either tubulin phosphorylation mediated by the endogenous microtubule-associated tubulin kinase (Fig. 8*A*, *light bars*) or rhodopsin phosphorylation mediated by purified GRK2 (Fig. 8*A*, *dark bars*). The addition of excess unlabeled GTP was used to determine the phosphoryl donor specificity of the endogenous tubulin kinase. Since GRK2 utilizes exclusively ATP (46), while casein kinase II can use both ATP and GTP as phosphate donors (45), GTP was utilized in this study to distinguish the activities of these two enzymes.

Both the microtubule-associated tubulin kinase and GRK2

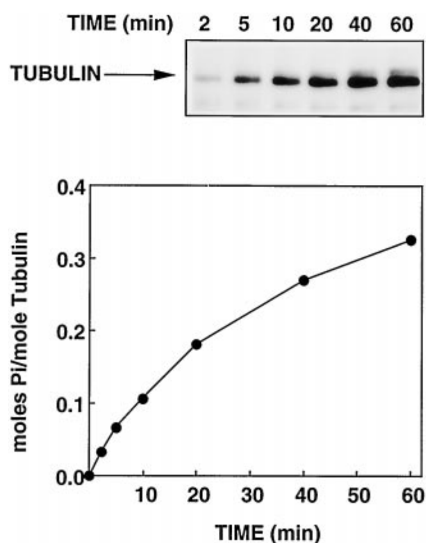

FIG. 7. **A tubulin kinase copurifies with microtubules.** Taxol-precipitated microtubules, prepared as described under "Experimental Procedures," were incubated with [γ-^{32}P]ATP for the times indicated. Reactions were quenched and subjected to electrophoresis, and gels were exposed to film (*upper panel*) and quantified using a PhosphorImager (*lower panel*). The results from a representative experiment are shown. Similar results were obtained with three different microtubule preparations. The migration position of tubulin is indicated on the autoradiograph.

exhibited very similar patterns of inhibitor sensitivity (Fig. 8*A*). Both enzymes were potently inhibited by heparin and unaffected following incubation with protein kinase A inhibitor, staurosporine, or excess GTP (Fig. 8*A*). The biochemical characteristics of the microtubule-associated tubulin kinase are thus consistent with its identification as GRK2.

Monoclonal antibodies directed against GRK2, GRK5, and GST were additionally utilized to characterize the microtubule-associated tubulin kinase. In the presence of anti-GRK2 antibodies, but not anti-GRK5 or anti-GST antibodies, tubulin phosphorylation mediated by the microtubule-associated kinase is inhibited by approximately 50%. Notably, under similar conditions these antibodies inhibit GRK2-mediated rod outer segment phosphorylation by approximately 75%. These results suggest that GRK2 is responsible for most of the tubulin kinase activity present in these microtubule preparations. Thus, three lines of evidence suggest that GRK2 is, at least in part, responsible for the endogenous microtubule-associated tubulin kinase activity: (i) GRK2 copurifies with microtubules, (ii) GRK2 exhibits similar biochemical characteristics to the endogenous tubulin kinase, and (iii) antibodies directed against GRK2 significantly inhibit the endogenous tubulin kinase activity. Considered together, these results suggest that GRK2 may play a physiological role as a tubulin kinase.

GRK2 Phosphorylates Tubulin in Vitro—The tubulin kinase activity of GRK2 was examined *in vitro* using purified proteins. Tubulin devoid of microtubule-associated proteins was purified using reversible, temperature-dependent assembly and phosphocellulose chromatography (25). As shown in Fig. 9, this highly purified tubulin preparation is essentially free of endogenous tubulin kinase activity (*open symbols*). The addition of purified GRK2 promotes rapid, stoichiometric phosphorylation of tubulin (Fig. 9, *closed symbols*). The maximal stoichiometry of phosphorylation approaches 1.0 mol of P_i/mol of tubulin, *i.e.* 2 mol P_i incorporated per mol of α/β heterodimer. Furthermore, the kinetic parameters for GRK2-mediated tubulin phosphorylation are similar to those for GRK2-mediated phosphorylation of agonist-occupied GPCRs, the only previously identified physiological substrates for GRK2. Table I lists the kinetic

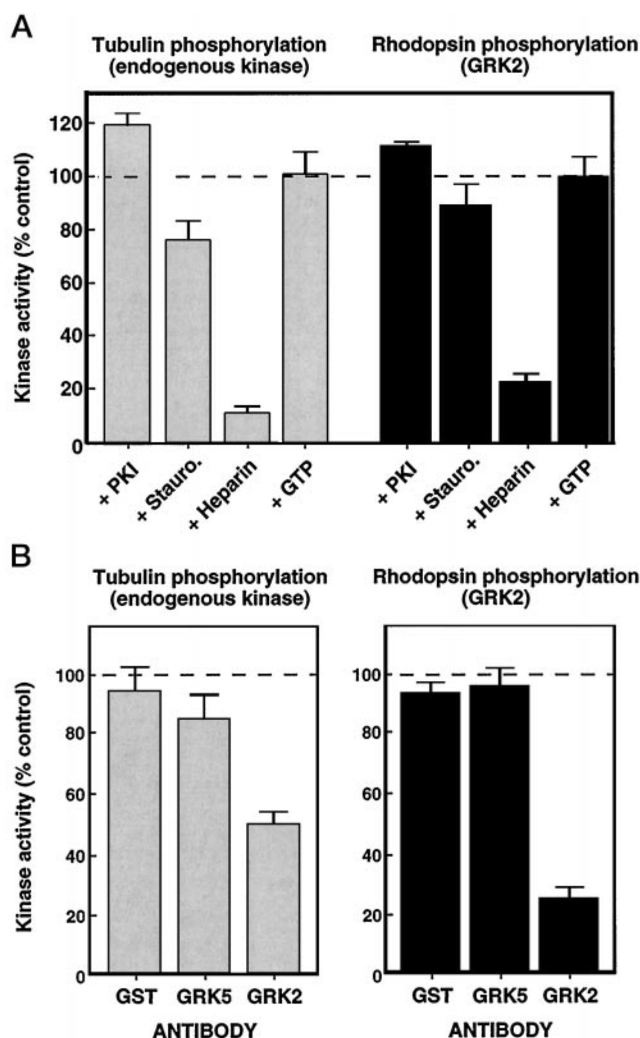

FIG. 8. **The endogenous tubulin kinase has biochemical properties similar to those of GRK2.** *A*, the effect of the protein kinase A inhibitor, staurosporine, heparin, and GTP on tubulin phosphorylation mediated by the microtubule-associated protein kinase (*light bars*) or rhodopsin phosphorylation mediated by GRK2 (*dark bars*) is shown. Assays were performed as described under "Experimental Procedures" for 10 min. 100% activity is that activity measured in the absence of inhibitors. The results shown represent the mean values ± S.E. for three separate determinations. *B*, the effect of monoclonal antibodies on tubulin phosphorylation mediated by the microtubule-associated protein kinase (*light bars*) or rhodopsin phosphorylation mediated by GRK2 (*dark bars*). Phosphorylation reactions were performed for 10 min in the presence of 10 μg of the indicated antibodies as described under "Experimental Procedures." 100% kinase activity is that measured in the absence of antibody addition. The results represent the mean values ± S.E. from three separate determinations using two different microtubule preparations.

parameters for GRK2-mediated phosphorylation of tubulin together with those for GRK2-mediated, isoproterenol-stimulated, β-AR phosphorylation. Notably, the K_m for GRK2-mediated tubulin phosphorylation is ~1.0 μM, while that for GRK2-mediated phosphorylation of the best peptide substrate is ~1340 μM (Table I and Ref. 31). Tubulin thus represents an approximately 1000-fold better substrate for GRK2 than peptide, suggesting that the tertiary structure of tubulin plays an important role in mediating the interaction with GRK2.

As described previously, $G_{\beta\gamma}$ subunits bind to and promote membrane association of GRK2, dramatically increasing the V_{max} for GRK2-mediated phosphorylation of membrane-incorporated GPCR substrates (10). As shown in Table I, the V_{max} for GRK2-mediated phosphorylation of the β-AR increases ap-

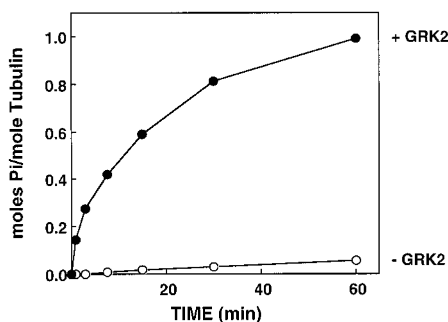

FIG. 9. **GRK2 phosphorylates tubulin.** Tubulin devoid of microtubule-associated proteins was incubated in the presence of ATP in either the absence (*open symbols*) or presence (*closed symbols*) of purified GRK2 (50 nM). Phosphorylation reactions were quenched at the indicated times. The results shown represent the mean values from two separate experiments.

TABLE I
Kinetic parameters of the GRK2 for β-AR, tubulin, and a soluble peptide substrate

Reconstituted β-AR (0.3–9.0 μM), purified tubulin (0.3–9.0 μM), or a soluble peptide substrate (10 μM to 1 mM) were phosphorylated as described under "Experimental Procedures." Phosphorylations were performed for 10 min at 30 °C, in either the presence or absence of the βγ-subunits of heterotrimeric G proteins (βγ, 200 nM). The results shown represent the mean values obtained from three separate determinations.

Substrate	Without βγ		With βγ	
	K_m	V_{max}	K_m	V_{max}
	μM	*pmol/min*	*μM*	*pmol/min*
β-AR	0.26	0.30	0.40	3.50
Tubulin	1.00	1.45	1.23	2.00
Peptide substrate (RRREEEEESAAA)	1340.00			

proximately 10-fold from 0.3 to 3.5 pmol of P_i/min upon the addition of $G_{\beta\gamma}$. In contrast, $G_{\beta\gamma}$ does not affect the kinetic parameters for GRK2-mediated phosphorylation of soluble tubulin. However, the V_{max} for tubulin phosphorylation (approximately 2.0 pmol of P_i/min) most closely approximates that for the β-AR in the presence of $G_{\beta\gamma}$. Tubulin thus represents the first non-GPCR substrate identified for GRK2, or indeed for any member of the GRK family. The similarity between the kinetic parameters of GRK2-mediated β-AR and tubulin phosphorylation *in vitro* suggests that, as with the β-AR, tubulin may represent a cellular substrate for this enzyme. Agonist occupancy of GPCRs recruits GRK2 in a $G_{\beta\gamma}$-dependent fashion to the plasma membrane (8). This $G_{\beta\gamma}$-dependent targeting of GRK2 to its receptor substrate activates the enzyme and promotes GRK2-mediated phosphorylation of nonreceptor (*i.e.* peptide) substrates *in vitro* (12, 13). Tubulin represents a potential candidate for such a non-GPCR GRK2 substrate *in vivo*.

Agonist Occupancy of GPCRs Promotes GRK2-tubulin Complex Formation and Tubulin Phosphorylation—Consistent with the model outlined above, agonist occupancy of GPCRs promotes GRK2-tubulin complex formation and tubulin phosphorylation in intact cells (Figs. 10 and 11). In HEK-293 cells transiently overexpressing GRK2, agonist occupancy of endogenously expressed GPCRs dramatically enhances the amount of tubulin associated with GRK2 as assessed by coimmunoprecipitation (Fig. 10). Activation of the β-AR, lysophosphatidic acid, and thrombin receptors increases the amount of tubulin present in GRK2 immunoprecipitations by approximately 8-fold. That this agonist-induced association of GRK2 and tubulin is accompanied by increased tubulin phosphorylation is shown in Fig. 11. Cells transiently overexpressing the β-AR were labeled with orthophosphate and incubated for 10 min in

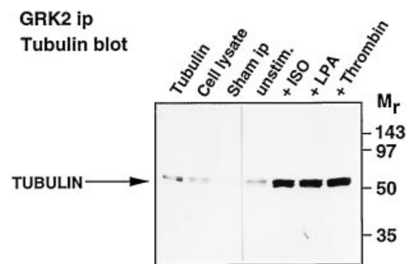

FIG. 10. **Activation of GPCRs facilitates the interaction of GRK2 with tubulin.** GRK2 was immunoprecipitated from HEK-293 cell lysates transiently overexpressing this enzyme. Cells were either unstimulated (*unstim.*) or treated with isoproterenol (+ *ISO*), lysophosphatidic acid (+ *LPA*), or the thrombin agonist peptide, SFLLRN, (+ *Thrombin*) for 5 min prior to harvest. GRK2 immunoprecipitates were subsequently subjected to Western blot analysis using an anti-tubulin antibody. Purified tubulin (tubulin) and 10 μg of HEK-293 cell lysate (*Cell lysate*) were used as positive controls. A control in which the immunoprecipitating antibody was omitted (*sham ip*) is also shown. The migration position of molecular weight standards and tubulin is indicated. The result shown is representative of three separate experiments.

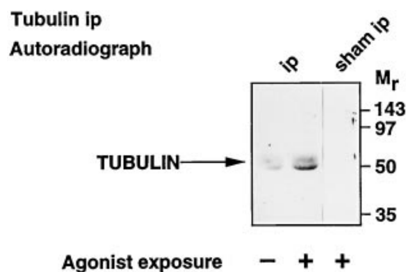

FIG. 11. **Tubulin is phosphorylated following β-AR activation.** HEK-293 cells transiently overexpressing GRK2 and β-AR were labeled with [^{32}P]orthophosphate as described under "Experimental Procedures." Cells were subsequently left unstimulated (−) or treated with isoproterenol for 10 min (+). Following harvest, a membrane fraction was prepared from which tubulin was immunoprecipitated. Tubulin immunoprecipitates were fractionated on SDS-polyacrylamide gels and exposed to film. A control in which the immunoprecipitating antibody was omitted is also shown (*sham ip*). The results shown are representative of two separate experiments.

the presence or absence of isoproterenol (a β-AR agonist). Tubulin was subsequently immunoprecipitated from either a whole cell lysate or a membrane fraction derived from these cells and immunoprecipitates subjected to autoradiography. Agonist occupancy of the β-AR promoted an approximately 2-fold increase in the ^{32}P content of total cellular tubulin (data not shown). More dramatically, however, an approximately 9-fold increase in the ^{32}P content of membrane-associated tubulin was observed upon GPCR activation (Fig. 11). That tubulin present in cellular membranes is specifically phosphorylated following β-AR activation may potentially be explained by the observations that (i) GRK2 associates with the plasma membrane following GPCR activation (8, 9) and (ii) that phosphorylated tubulin preferentially associates with lipid vesicles (47).

GRK2-mediated Tubulin Phosphorylation: Potential Physiological Significance—GRK2-mediated β-AR phosphorylation plays a critical role in mediating rapid agonist-induced receptor desensitization (reviewed in Refs. 1–3) and targets the β-AR for internalization (7). Does this enzyme have additional cellular functions? In this report we demonstrate that GRK2 associates with microtubules and with soluble tubulin in a cellular extract and in living cells and that tubulin represents an excellent substrate for this enzyme *in vitro*. Notably, the kinetic parameters of GRK2-mediated tubulin phosphorylation mirror those of GRK2-mediated β-AR phosphorylation, a physiological sub-

strate of this enzyme, and far surpass those of peptide substrates. Agonist occupancy of GPCRs promotes GRK2-tubulin complex formation and tubulin phosphorylation. Taken together, these observations suggest a potential role for GRK2 in modulating the phosphorylation status of tubulin in intact cells.

The interaction of GRK2 with activated receptor substrates *in vitro* leads to an allosteric activation of this enzyme (12, 13). The physiological relevance of this activation has remained somewhat obscure, since to date the only substrates identified for these enzymes were activated receptors themselves. The identification of a potential non-GPCR GRK2 substrate raises the possibility that GRK2 may itself act as a signal transducer. Agonist occupancy of GPCRs promotes the membrane localization of GRK2 and, specifically, the targeting of this enzyme to its activated receptor substrates. Allosteric activation of GRK2 by activated receptors would thus be predicted to promote GRK2-mediated phosphorylation of tubulin in an agonist-stimulated manner. In such a model, GRK2 would act to intimately link GPCR activation with one component of the cellular cytoskeleton.

Notably, several studies utilizing fluorescently labeled β-AR antagonists have revealed that the receptors are motionally constrained within the plasma membrane. The constraining influence appears to be mediated largely via interactions of the receptor with microtubules, since agents like colchicine, but not cytochalasin D, relieve the constraint and increase receptor mobility (48, 49). Moreover, occupancy of the receptor by the agonist isoproterenol but not the antagonist propranolol similarly releases the constraint on receptor mobility. It is tempting to speculate that GRK2 may play a role in mediating this agonist-stimulated dissociation of the β-AR from cytoskeletal elements.

Microtubules have been implicated as playing a role in mediating internalization of the β-AR (50). In this regard, it is interesting to note that AP3, a synapse-specific clathrin adaptor protein that promotes clathrin cage formation, has been reported to bind dephosphorylated tubulin but not tubulin phosphorylated by an unidentified tubulin-associated kinase (42). GRK2 is tightly associated with microtubules and phosphorylates tubulin *in vitro*. The potential role of GRK2-mediated tubulin phosphorylation in regulating β-AR internalization remains to be elucidated. Tubulin represents the first identified non-GPCR substrate for GRK2, an observation that hints that the physiological role(s) of GRK2 may be more diverse than currently appreciated.

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