Crystal Structure of the cGMP-dependent Protein Kinase II Leucine Zipper and Rab11b Protein Complex Reveals Molecular Details of G-kinase-specific Interactions**

Received for publication, April 29, 2014, and in revised form, July 21, 2014. Published, JBC Papers in Press, July 28, 2014, DOI 10.1074/jbc.M114.575894

Albert S. Reger†, Matthew P. Yang§, Shizuyo Koide-Yoshida¶, Elaine Guo‖, Shrenik Mehta‡, Keizo Yuasa§, Alan Liu**, Darren E. Casteel**, and Choel Kim‡‡*

From the †Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030, the Departments of ‡Biochemistry and Chemistry, Rice University, Houston, Texas 77005, the ‡Department of Biomedical Science and Technology, University of Tokushima Graduate School, Tokushima 770-8506, Japan, the **Department of Medicine, University of California at San Diego, La Jolla, California 92093, and the ‡‡Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, Texas 77030

Background: cGMP-dependent protein kinases utilize their leucine zipper (LZ) domains to bind interacting proteins in an isotype-specific manner.

Results: Structural and biophysical analysis reveals residues for the PKG II-Rab11b interaction.

Conclusion: PKG II utilizes an electroneutral surface on the LZ domain to bind Rab11b.

Significance: This is the first structure of PKG bound to one of its interacting proteins.

Cyclic guanosine monophosphate (cGMP) is a crucial second messenger that relays extracellular signals to various effectors inside the cell. As the main receptor for cGMP, cGMP-dependent protein kinase (PKG)-interacting proteins (GKIPs) mediate cellular targeting of PKG isoforms by interacting with their leucine zipper (LZ) domains. These interactions prevent aberrant signaling cross-talk between different PKG isotypes. To gain detailed insight into isotype-specific GKIP recognition by PKG, we analyzed the type II PKG leucine zipper isotypes. To gain detailed insight into isotype-specific GKIP recognition by PKG, we analyzed the type II PKG leucine zipper domain and found that residues 40–83 dimerized and specifically interacted with Rab11b. Next, we determined a crystal structure of the cGMP-dependent protein kinase (PKG)-interacting proteins (GKIPs) mediate cellular targeting of PKG isoforms by interacting with their leucine zipper (LZ) domains. These interactions prevent aberrant signaling cross-talk between different PKG isotypes. To gain detailed insight into isotype-specific GKIP recognition by PKG, we analyzed the type II PKG leucine zipper domain and found that residues 40–83 dimerized and specifically interacted with Rab11b. Next, we determined a crystal structure of the PKG II LZ-Rab11b complex. The PKG II LZ domain presents a mostly nonpolar surface onto which Rab11b docks, through van der Waals interactions. Contact surfaces in Rab11b are found in switch I and II, interswitch, and the B1/N-terminal regions. This binding surface dramatically differs from that seen in the Rab11 family of interacting protein complex structures. Structural comparison with PKG Iα and Iβ LZs combined with mutagenic analysis reveals that GKIP recognition is mediated through surface charge interactions.

† This work was supported, in whole or in part, by National Institutes of Health Grant R01 GM090161 (to C. K.).
‡ This article was selected as a Paper of the Week. The atomic coordinates and structure factors (code 4OJK) have been deposited in the Protein Data Bank (http://wwpdb.org/).
§ To whom correspondence should be addressed: MS:330 Alkek 520, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Tel.: 713-798-8411; Fax: 713-745-2107; E-mail: ckim@bcm.edu.
¶ The abbreviations used are: GKIP, PKG-interacting protein; LZ, leucine zipper; RBD, Rab11-binding domain; PDB, Protein Data Bank; CFT, cystic fibrosis transmembrane conductance regulator; FIP, family of interacting protein; TEV, tobacco etch virus; ITC, isothermal titration calorimetry; VDW, van der Waals; IMAC, immobilized metal ion affinity chromatography; GMPPNHP, guanosine 5’-[β,γ-imido]triphosphate.
PKG II-Rab11b Complex Structure

phorylation of the inositol triphosphate receptor-associated PKG substrate by PKG inhibits Ca^{2+} release from the 1,4,5-
inositol triphosphate receptor, which also contributes to smooth muscle relaxation (11, 12). PKG II phosphorylates and
activates the cystic fibrosis transmembrane conductance regu-
lator (CFTR) in intact cells (13, 14). CFTR is a good in vitro
substrate for both PKG II and PKG Iβ, but PKG Iβ is unable to
activate CTFR in intact cells. However, a PKG Iβ chimera con-
taining the membrane targeting domain of PKG II (residues
1–29) activates CFTR, although only 30–40% as effective as
PKG II. These results suggest that other regions of PKG II are
involved in targeting it to CFTR. Although these data show that
each isoform of PKG interacts with isoform-specific GKIPs,
which mediates specific subcellular localization and provides a
mechanism for substrate specificity, little is known about the
details of these interactions due to the lack of structural informa-
tion (13, 14).

Rab11 is a subfamily of the Ras small GTPases and includes
Rab11a and Rab11b, which share 89% sequence identity with
each other, and Rab25, which has 61 and 66% sequence identity
to Rab11a and Rab11b, respectively (15). Although Rab11a and
Rab11b are ubiquitously expressed, Rab25 is found exclusively
in epithelial cells (16–18). Rab11 plays a major role in the main-
tenance of the slow recycling endosome pathway and traffick-
ing cargo to the plasma membrane. Structurally, the small
GTPase family has a conserved G protein fold consisting of a
six-strand β-sheet flanked by α-helices on each side. Two struc-
turally conserved motifs of the Ras family, the switch I and II
regions, play a role in specifically binding downstream effectors
and modulating effector affinity by having two distinct func-
tional states. The GTP-bound state is considered the “on” state
and has high affinity for downstream effectors; the GDP-bound
state is considered the “off” state with low affinity (19, 20). A
major focus of research on Rab11 signaling has been to study its
interaction with the five members of the Rab11 family of interact-
ing proteins (FIPs) (21). The FIPs form homodimers through a
C-terminal leucine zipper, which functions as a Rab11-bind-
ing domain (RBD). FIPs preferentially interact with GTP-
bound Rab11 (22–25). The interactions between Rab11 and
FIPs are essential in regulating recycling endosome trafficking
and delivery of cargo to specific locations on the plasma mem-
brane (21).

Reports have shown that Rab11 can interact with several
membrane-associated proteins. Specifically, it was shown that
PKG II interacts with GDP-bound Rab11b and that this inter-
action is crucial for their co-localization at the recycling endo-
some and their subsequent return to the plasma membrane
(26). Rab11 has also been reported to interact with other mem-
brane-associated proteins such as the β2-adrenergic receptor,
TRPV5 and TRPV6 Ca^{2+} channels, β-isomer of the thrombox-
cane A_{2} receptor (TPB), and brain-derived neurotrophic factor-
dependent TrkB (TrkB-FL) receptors (27–30).

To understand the molecular details of the PKG II-Rab11b
interaction, we identified a PKG II LZ fragment that stably
bound Rab11b and determined a crystal structure of their com-
plex. Our structure of the PKG II-Rab11b complex, combined with
mutagenic analysis, reveals the molecular details of the
PKG II-Rab11b interaction and provides the structural insight
into the isotype-specific GKIP-PKG interactions.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—PKG II LZ (residues
40–83) was inserted into the expression vector pQTEV with an
N-terminal His_{6} tag and transformed into BL21 (DE3) Esche-
richia coli cells (31). Cells were grown at 37 °C and induced with
0.5 mM isopropyl 1-thio-β-D-galactopyranoside at A_{600} = 0.5.
The cells continued to grow for 18 h at 18 °C after induction.
The cell pellet was suspended in Lysis Buffer A (50 mM Tris (pH
7.5), 200 mM NaCl, 5 mM MgCl_{2}, and 5 mM imidazole) and
lysed with Constant Systems TS cell disrupter (Daventry Northants,
UK). The lysate was cleared using ultracentrifugation, and the
supernatant was loaded onto an IMAC nickel column. The col-
umn was washed with Lysis Buffer A, and the sample was eluted
in Lysis Buffer A containing 300 mM imidazole. His-tagged
tobacco etch virus (TEV) protease was added to cleave the His
tag, and the cleaved sample was again loaded onto an IMAC
nickel column for TEV separation. The sample was concen-
trated and loaded onto a HiLoad 16/60 Superdex 75 gel filtra-
tion column (GE Healthcare) equilibrated with 25 mM Tris
(pH 7.5), 25 mM NaCl, and 1 mM tris(2-carboxyethyl)phosphine.

Using a protocol similar to the one above, Rab11b (residues
8–205) was inserted into the expression vector pQTEV and
expressed in BL21 (DE3) E. coli cells (31). Cells were grown at
37 °C, induced with 0.5 mM isopropyl 1-thio-β-D-galactopy-
ranoside at A_{600} = 0.6, and continued to grow for 4 h at 37 °C after
induction. The cell pellet was suspended in Lysis Buffer B (50
mM Tris (pH 7.5), 50 mM NaCl, 5 mM β-mercaptoethanol)
and cleared using ultracentrifugation. The supernatant was
loaded onto an IMAC nickel column, and the column was washed
with Lysis Buffer B, and the sample was eluted with Lysis Buffer B
containing 300 mM imidazole. TEV was added to cleave the His
tag. Cleaved sample was loaded again onto an IMAC nickel
column for TEV separation. The sample was concentrated and
loaded onto a HiLoad 16/60 Superdex 75 gel filtration column
(GE Healthcare) equilibrated with 25 mM Tris (pH 7.5), 25 mM
NaCl, 1 mM MgCl_{2}, and 0.5 mM tris(2-carboxyethyl)phosphine.

In Vitro Pulldowns—GST and GST-tagged PKG leucine zip-
per domains were expressed in E. coli and bound to glutathione-Sepharose beads as described (32). Binding reactions con-
tained 10 µg of GST or each GST-tagged leucine zipper
incubated with 10 µg of Rab11b in 200 µL of Binding Buffer
(50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM MgCl_{2}, 0.1 mM EDTA,
0.1 mM EGTA, 0.2% BSA, and 0.1% Triton X-100). The reac-
tions were incubated at 4 °C for 1 h. The beads were washed
three times with the binding buffer, and bound proteins were
analyzed by SDS-PAGE followed by immunoblotting with anti-
His and anti-GST antibodies (Santa Cruz Biotechnology).

Crystallization and Structure Solution—Prior to crystalliza-
tion, the protein complex was formed by mixing an equal molar
ratio of Rab11b and PKG II LZ along with 5 mM MgCl_{2} and 10
mM guanosine diphosphate (GDP) to a final concentration of 3
mM. Crystals were obtained by mixing in a 1:1 ratio of protein
and 0.056 M sodium phosphate monobasic monohydrate, 1.344
M potassium phosphate dibasic (pH 8.2), and 10 mM EDTA
PKG II LZ Binds Directly to Rab11b in a GDP-dependent Manner—PKG II was previously found to interact with Rab11b using the yeast two-hybrid system and co-immunoprecipitation from transiently transfected cells (26). Alanine mutations within the dimeric interface of the PKG II LZ were shown to disrupt Rab11b association, suggesting that the PKGII LZ domain mediates the interaction; however, direct binding between the proteins was not shown (26). We analyzed the N-terminal region of PKG II using the program 2ZIP to identify boundaries of the heptad repeat motif common in the LZ domains, which defined the PKG II LZ to be residues 40–83 (Fig. 1B) (39). To test whether Rab11b and the PKG II LZ interact directly, we performed in vitro pulldowns using His-tagged Rab11b (residues 8–205) and GST-tagged PKG Iα, Iβ, or II LZ domains immobilized on glutathione-Sepharose beads. As shown in Fig. 1C, Rab11b bound specifically to the PKG II LZ. Under these conditions, no interaction was seen between Rab11b and GST or the PKG Iα/PKG Iβ LZs (Fig. 2C). Next, we analyzed the PKG II LZ-Rab11b interaction using isothermal titration calorimetry (ITC). ITC measurements showed $K_d$ values ranging 22–41 μM for the PKG II LZ. Interestingly, the $K_d$ value was slightly lower when Rab11b was bound to GDP compared with the GTP (22 μM versus 41 μM) (Fig. 1, D and E). When the PKG Iα or Iβ LZ was mixed with Rab11b, we did not detect a heat exchange signature suggesting that neither PKG I isoform interacts with Rab11b in solution (data not shown).

PKG II-Rab11b Complex Structure—To gain structural insight into how the PKG II LZ domain interacts with Rab11b, we determined the crystal structure of the PKG II LZ-Rab11b complex (Fig. 2 and Table 1). The structure was determined to 2.66 Å with the asymmetric unit containing two molecules of PKG II LZ (referred as LZ and LZ) and two molecules of Rab11b (referred to as Rab11b and Rab11b'). The asymmetric unit contains a Rab11b molecule on either side of the PKG II LZ (Rab11b, LZ-LZ', and Rab11b'). The PKG II LZ mainly interacts with the switch I, II, intershwitch, and the β1/β-terminal regions of Rab11b (Fig. 2A).

Overall Structure of the PKG II LZ Domain—The structure shows that the PKG II LZ forms a parallel coiled-coil, with the heptad repeats placing the hydrophobic portion of the amphiphilic helices in the dimerization interface (Figs. 2A and 3). Although the purified protein contained residues 40–83 corresponding to six heptad repeats, the first heptad repeat was not modeled due to lack of electron density. Seven N-terminal residues are missing in one of the chains (LZ, residues 47–83), although two additional residues are missing at either terminus for the other chain (LZ', residues 49–81).

With exception of Glu-61 and Lys-75, leucine or isoleucine residues at the $a$ and $d$ positions of heptad repeats of each monomer pack in a “knob-into-holes” manner forming an extensive hydrophobic core with a 735 Å$^2$ surface area. Interestingly, the side chain of Glu-61 at the $d$ position from one monomer forms a hydrogen bond with the side chain of Thr-62' at the e position of the other monomer (Fig. 3). The interhelical hydrogen bond between Glu-61 and Thr-62' forms in a symmetrical manner. The $d$ position Lys-75 shows no interhelical salt bridges.

The surface of PKG II LZ domain shows two distinct surfaces: a charged surface with negative and positive patches near the N and C termini, respectively, and a hydrophobic surface at the center of the LZ, which provides most of the docking surface for Rab11b. Superhelical and α-helical parameters were
analyzed using Twister showing similar values as the previously reported coiled-coil structures, including PKG Iα LZ (40, 41). Both strands exhibit higher temperature factors toward the N and C termini, suggesting increased thermal motion or flexibility in those regions, and show minor distortion based on the α-helical parameters by calculated Twister (41). LZ and LZβ, superimpose well with a root mean square deviation of 0.89 Å for 30 equivalent Cα atoms between residues 49 and 81. The N terminus of LZβ shows a slight kink that may be caused by crystal packing or by its interaction with Rab11b.

Rab11b Structure—As mentioned, the asymmetric unit of the crystal contained two molecules of Rab11b (Rab11b and Rab11bβ) both with GDP bound (Fig. 4). Superposition of each molecule with the previously determined Rab11b-GDP structure gives a root mean square deviation of 0.92 Å for 30 equivalent Ca atoms between residues 49 and 81. The N terminus of LZβ shows a slight kink that may be caused by crystal packing or by its interaction with Rab11b.

PKG II–Rab11b Complex Interface—The crystal structure of the complex reveals that PKG II uses a total of four heptad repeats to bind Rab11b (Fig. 6A). Residues 54–83 of each PKG II LZ interact with both molecules of Rab11b mainly through hydrophobic interactions. Because of disorder in the switch I and II regions in Rab11bβ, the overall interaction surface is not the same between the two chains of the LZ. The total interface surface area between the Rab11b molecule (with the ordered switch regions) and PKG II LZ is 1078 Å². The interface surface area for the less ordered Rab11bβ is 855.8 Å². The PKG II LZ docks to multiple regions of Rab11b, including switch I and II, the interswitch, and the N1/N-terminal regions (Fig. 6B). The first interface forms between the N-terminal region of the PKG II LZ and switch I of Rab11b and, as mentioned above, shows little symmetry in the interaction pattern due to the par-
tially disordered regions in Rab11b’ (upper panel of Fig. 6B). Interestingly, the core residues (Leu-58 from one strand and Gln-61 from the other at the a and d positions) of the LZ docks to switch I (residues 44–48) mainly through van der Waals (VDW) interactions. A single hydrogen bond forms between Gln-57 NE2 of PKG II LZ and the backbone carbonyl oxygen of Gly-45 of Rab11b at this interface.

The second interface forms between the middle section of the LZ (residues 69–83) and switch II of Rab11b and involves mainly VDW contacts, with the following two exceptions: a salt bridge between side chains of Glu-70 of the PKG II LZ and Arg-82 of Rab11b and a hydrogen bond between the side chain of Gln-73 of LZ and the backbone carbonyl of Gly-83 at the end of switch II (lower panel of Fig. 6B). The hydrophobic interface at this region can be broken into two parts. The first part is the aromatic ring of Tyr-73 of the Rab11b docking to a hydropho-

---

**FIGURE 2. Crystal structure of the PKG II LZ-Rab11b complex.** A, structure of the PKGII LZ-Rab11b complex with the secondary structure elements labeled. The disordered switch I and II regions of Rab11b’ are shown as a dotted lines. All structure images were generated using PyMOL (Delano Scientific). B, surface representation of the complex is shown, colored according to its electrostatic potential (red, electronegative; blue, electropositive).

**FIGURE 3. Structure of the PKG II LZ domain.** The structure of the PKG II LZ domain showing the overall dimensions in angstroms (Å), with stick representation of the side chains from each monomer. Only one of the chains is shown with surface. Inset, cutaway view looking down the 2-fold axis. This view shows the symmetrical hydrogen bond formed by Gln-61 of one chain and Thr-62 of the other.

---

**TABLE 1 Data and refinement statistics**

| PKG II LZ-Rab11b-GDP |
|----------------------|
| **Data collection**   |
| Space group          | *P*4_2_2 |
| Cell dimensions      | a, b, c (Å) | 136, 136, 76.9 |
| α, β, γ (°)          | 90, 90, 90 |
| Resolution (Å)       | 19.9–2.66 (2.8–2.66) |
| Rsym (%)             | 9.3 (3.5) |
| Completeness (%)     | 99.2 (99.5) |
| Redundancy           | 14.2 (13.9) |
| **Refinement**       |
| Resolution           | 19.9–2.66 (2.77–2.66) |
| No. of unique reflections | 21,139 (3030) |
| R_work/R_free        | 20.1/25.1 (33.9/38.7) |
| **B-factors**        |
| Protein (chain)      | 62.6(A), 73.6(B), 74.1(C), 76.8(D) |
| Ligand               | 57.3 |
| Water                | 57.9 |
| **Root mean square deviations** |
| Bond lengths (Å)     | 0.003 |
| Bond angles (°)      | 0.694 |
| **Ramachandran plot (%)** |
| Most favorable region| 96.9 |
| Additional allowed region | 3.1 |
| Outliers             | 0 |

*a* Highest resolution shell is shown in parentheses.

*b* 5.0% of the observed intensities were excluded from refinement for cross-validation purposes.
bic cleft formed by Arg-55, Leu-58, Ala-59, and Leu-54’ of the PKG II LZ. The second part involves the side chain of Ile-76 and Ala-59 from Rab11b docking onto another hydrophobic patch consisting of Thr-62 and Val-63 of the PKG II LZ. These interactions induce major structural changes in switch II, which involves the 3_10-helix and a2-helix moving 10 Å away from the core toward the interaction interface (Fig. 4B). The structural rearrangement exposes the buried side chain of Ile-76 for its interaction with PKG II LZ (Fig. 5B).

The third interface forms between the C-terminal end of the LZ and the continuous surface formed between the interswitch, strand β1, and N-terminal regions of Rab11b (Fig. 6B). This region makes up over 50% of the total interface. Although the majority of the contacts are through VDW interactions, four hydrogen bonds are also present at this interface. PKGII Thr-69 contributes two hydrogen bonds through its interaction with the side chain of Gln-63 of Rab11b, and the side chains of PKGII Gln-73 and Asn-80 form hydrogen bonds with the Rab11b backbone amide of Lys-13 and the carbonyl of Asp-9, respectively. Another hydrogen bond was formed between the side chain of PKGII Glu-71 and the hydroxyl group of Tyr-8 of

FIGURE 4. Electron density of GDP bound to Rab11b. Unbiased ligand density is shown for Rab11b; the electron density maps were calculated with coefficients of the form $F_o - |F_c|$ determined prior to insertion of GDP in the refinement. The maps are contoured at 4σ, and the side chains of neighboring residues are labeled.

FIGURE 5. Structure of Rab11b. A, stereo image of the superposition of Rab11b (red), Rab11b’ (black), and the previously solved Rab11b-GDP complex (blue) (PDB code 2F9L) (36). B, structural alignment of the PKG II LZ-Rab11b (red) with the previously determined structures of Rab11b bound to GMPNP and GDP in teal (PDB code 2F9M) and GDP in purple (PDB code 2F9L) (36). The enlarged panel to the right shows a close-up view of the rearrangement of the 3_10-helix near the switch II region of Rab11b, which places Tyr-73 and Ile-76 in position to make contacts with the PKG II LZ.

FIGURE 6. Interface of the PKG II LZ-Rab11b complex. A, color-coded representation of the regions on Rab11b and PKG II that form the interaction interface. The coloration corresponds to the Rab11b surfaces that interact between the two proteins: switch I (red), switch II (magenta), interswitch (red), and N terminus (yellow), B, PKG II LZ-Rab11b interactions with zoom-in views. Rab11b provides the largest interface at the interswitch/N-terminal region, contributing van der Waals contacts from residues Asp-9 to Phe-12, Phe-48, Lys-58, Lys-61, Trp-65, and Val-85. PKG II LZ provides residues Thr-62, Ile-65, Ala-66, Leu-72, Gln-73, Cys-76, Ile-77, and Lys-81 of PKG II LZ to this interface. Dashed lines in the insets represent hydrogen bonds and salt bridge interactions.
FIGURE 7. Representation of mutations made to Rab11b, mutant ITC measurements, and subcellular localizations of PKG II and Rab11b in HeLa cells. A, schematic representation of the PKGII LZ-Rab11b complex with enlarged panels showing the interface seen in the crystal structure (left) and in a modeled structure with PKG II T62E/A66Q mutations (right). B and C, ITC was performed as described under “Experimental Procedures.” No exothermic response was seen when titrating Rab11b into PKG II LZ with a T62E mutation (B) or A66Q mutation (C). D, HeLa cells expressing PKG II-FLAG (panels 1–3) or PKG II T62E/A66Q-FLAG (panels 4–6) together with HA-Rab11b were analyzed by confocal microscopy, as described under “Experimental Procedures.” PKG II WT-FLAG (panel 1, green) and HA-Rab11b (panel 2, red) co-localized in the recycling compartment (panel 3, areas of colocalization are yellow), whereas PKG II T62E/A66Q-FLAG did not co-localize with HA-Rab11b (panels 4–6).
Rab11b. Unlike the asymmetrical interactions at switch I and II, the interactions at the third interface are highly similar for both Rab11b and Rab11b/H11032 molecules in the asymmetric unit and show clear electron density for all contact residues.

**T62E or A66Q Mutations in PKG II LZ Disrupt Rab11b Interaction**—To verify our structural results, we mutated key Rab11b contact residues within the PKG II LZ (Thr-62 and Ala-66) and measured their affinities of the mutants for Rab11b using ITC (Fig. 7). Based on our sequence alignment (Fig. 1B), we mutated Thr-62 and Ala-66 to the corresponding residues (Glu and Gln) in PKG Iα. Because Thr-62 and Ala-66 are located at the center of the PKG II LZ-Rab11b interface, we reasoned that the mutations would destabilize their interaction by changing the surface contour and the overall charge distribution of the binding surface (Fig. 7A). Indeed, our ITC data showed a lack of exothermal response upon injection indicating that either mutation in the PKG II LZ abolished its interaction with the Rab11b (Fig. 7B).

**T62E/A66Q Mutation in PKG II Disrupts PKG II and Rab11b Interaction in Vivo**—Next, we introduced the T62E/A66Q mutations into full-length PKG II and tested to see whether these mutations would disrupt the interaction with Rab11b in mammalian cells. We co-transfected HeLa cells with Rab11b and full-length wild-type PKG II or T62E/A66Q mutant PKG II and looked for co-localization using immunofluorescence microscopy. In cells with wild-type PKG II, Rab11b and PKG II were co-localized in a punctate staining pattern (Fig. 7D, panels 1–3), consistent with the known association of Rab11b with recycling endosomes (43). However, cells transfected with the T62E/A66Q mutant PKGII (43) showed a completely different staining pattern (Fig. 7D, panels 4–6), where PKG II localized to the plasma membrane and pericentriolar region and showed very little co-localization with Rab11b. In addition, Rab11b showed a diffuse staining pattern in PKG II T62E/A66Q mutant-expressing cells, suggesting that the mutant PKG II may influence the distribution of Rab11b. These results demonstrated that the T62E/A66Q mutations in the full-length PKG II abolish its interaction with Rab11b in vivo, further supporting our structural results.

**DISCUSSION**

Although PKG LZ-GKIP interactions are known to play key roles in mediating PKG signaling, to this point there has been no detailed structural insight into how the PKG isotypes utilize their LZ domains to recognize GKIPs. Our PKG II LZ-Rab11b complex represents the first crystal structure of the LZ domain of PKG II, and the first structure of a PKG-GKIP complex. With this structure, we provide new insight into how the PKG II LZ provides an isotype-specific docking surface for Rab11b and reveal a new protein-protein interaction surface in Rab11b.

Structural comparison with other LZ domains shows that the surface of PKG II LZ is drastically different in its charge distri-
bution and surface contour. Structures of LZs are reported for both PKG I/H9251 (PDB 1ZXA) and I/H9252 (PDB 3NMD) (40, 44). As seen in the Fig. 8, the PKG II LZ displays a large uncharged surface at the center. In major contrast, the I/H9251 LZ shows both positively and negatively charged regions across its LZ domain, whereas the I/H9252 LZ shows a largely electronegative surface throughout.

Alignment of the PKG II LZ with both the PKG I LZs shows that the regions that overlap with the Rab11b docking surface within PKG II LZ display a very different charge distribution and a unique contour suggesting that these surfaces do not provide a compatible surface for the Rab11b binding. We exploited these differences in selecting which PKG II residues to mutate to disrupt the PKG II LZ and Rab11b interaction. Indeed, subsequent mutagenic analysis combined with ITC and co-localization experiments shows that mutating Thr-62 and Ala-66 residues of PKG II LZ disrupt the interaction in vitro and in vivo.

Structural alignments between the PKG LZs, combined with mutagenesis/binding data, suggest that both surface charge distribution and topology are crucial in isotype-specific PKG-GKIP interactions. Based on our structural analysis of the PKG II LZ-Rab11b interaction, we predict that PKG I/H9251 and I/H9252 will predominantly recognize their isotype-specific GKIPs via hydrogen bond and salt bridge interactions. In conclusion, we present the first crystal structure of a PKG and GKIP complex. This structure has provided structural insight into the direct interaction between PKG II and Rab11b.

FIGURE 9. Structural comparison of the PKG II LZ-Rab11b complex with Rab11a-FIP complexes. A, cartoon representations of the Rab11b-PKG II LZ complex, Rab11a-FIP2 (PDB code 2HV8) (25), and Rab11a-FIP3 (PDB code 2GZD) (24) with PKG II LZ and FIPs aligned. B, complexes aligned with molecules of Rab11. Note that the FIP binding region is mainly along switch I and II of Rab11a, whereas PKG II LZ interacts mainly in the interswitch and N-terminal region of Rab11b. C, view showing only the PKG II LZ; FIP2, and FIP3 highlights the steric clashes.

In the GTP-bound state, switch II directly interacts with GTP, and its conformation appears fixed (36). In contrast, in the Rab11b GDP structure, switch II does not interact with GDP, and the structure shows higher temperature factors for the residues in this region suggesting it is flexible (36). This flexibility allows switch II to make a direct interaction with the PKG II LZ. However, in the GTP-bound state, switch II is pulled away from the LZ interface, and it no longer interacts with the LZ domain. The switch II interaction is made up of relatively weak VDW contacts, which contribute little to the overall interface. Nevertheless, these contacts may explain the increased affinity in the GDP-bound state.

In conclusion, we present the first crystal structure of a PKG and GKIP complex. This structure has provided structural insight into the direct interaction between PKG II and Rab11b.
Our comparative structural analysis combined with biochemical and co-localization data has shown that the drastically different surface charge distribution of each leucine zipper domain is likely to be one of the major determinants for the isoform-specific PKG-GKIP interactions; these interactions are required for PKG subcellular localization and ensure signaling fidelity by limiting cross-talk between different PKG isoforms. It will be interesting to further investigate how other PKG I isoforms use the more electrostatically charged LZ surface to recognize interacting proteins.

Acknowledgments—We thank B. Goud, C. Peters, G. Y. Huang, T. Palzkill, and J. J. Kim for critical reading of the manuscript. We appreciate the technical support, advice, and assistance of D. C. Chow and K. Klerc in the Center for Drug Discovery at Baylor College of Medicine. We also thank S. R. Wasserman (Eli Lilly Beamline, Advanced Photon Source) for assistance with data collection. Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Department of Energy Office of Science by Argonne National Laboratory, was supported by the United States Department of Energy under Contract DE-AC02-06CH11357. Use of the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at Sector 31 of the Advanced Photon Source was provided by Eli Lilly Co., which operates the facility.

REFERENCES

1. Hofmann, F., Bernhard, D., Lukowski, R., and Weinmeister, P. (2009) cGMP-regulated protein kinases (cGK). Handb. Exp. Pharmacol. 191, 137–162
2. Hofmann, F., Feil, R., Kleppisch, T., and Schlossmann, J. (2006) Function of cGMP-dependent protein kinases as revealed by gene deletion. Physiol. Rev. 86, 1–25
3. Francis, S. H., Busch, J. L., Corbin, J. D., and Sibley, D. (2010) cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. Pharmacol. Rev. 62, 525–563
4. Orstavik, S., Natarajan, V., Taskén, K., Jahnson, T., and Sandberg, M. (1997) Characterization of the human gene encoding the type Iα and type Iβ cGMP-dependent protein kinase (PRKG1). Genomics 42, 311–318
5. Sandberg, M., Natarajan, V., Ronander, I., Kalderon, D., Walter, U., Lohmann, S. M., and Jahnson, T. (1989) Molecular cloning and predicted full-length amino acid sequence of the type Iβ isozyme of cGMP-dependent protein kinase from human placenta. Tissue distribution and developmental changes in rat. FEBS Lett. 255, 321–329
6. de Jonge, H. R. (1981) Cyclic GMP-dependent protein kinase in intestinal brush borders. Adv. Cyclic Nucleotide Res. 14, 315–333
7. Pfeffer, S. R. (2005) Structural clues to Rab GTPase functional diversity. J. Biol. Chem. 280, 15465–15488
8. Horgan, C. P., and McCaffrey, M. W. (2009) The dynamic Rab11-FIPs. Biochem. Soc. Trans. 37, 1032–1036
9. Shiba, T., Koga, H., Shin, H. W., Kawasaki, M., Kato, R., Nakayama, K., and Wakatsuki, S. (2006) Structural basis for Rab11-dependent membrane recruitment of a family of Rab11-interacting protein 3 (FIP3)/Arfophilin-1. Proc. Natl. Acad. Sci. U.S.A. 103, 15416–15421
10. Junutula, J. R., Schonteich, E., Wilson, G. M., Peden, A. A., Scheller, R. H., and Prekeris, R. (2004) Molecular characterization of Rab11 interactions with members of the family of Rab11-interacting proteins. J. Biol. Chem. 279, 33430–33437
11. Jagoe, W. N., Lindsay, A. J., Read, R. J., McCoy, A. J., McCaffrey, M. W., and Khan, A. R. (2006) Crystal structure of rab11 in complex with rab11 family interacting protein 2. Structure 14, 1273–1283
12. Eathiraj, S., Mishra, A., Prekeris, R., and Lambright, D. G. (2006) Structural basis for Rab11-mediated recruitment of FIP3 to recycling endosomes. J. Mol. Biol. 364, 121–135
13. Yuasa, K., Yamagami, S., Nagahama, M., and Tsuji, A. (2008) Trafficking of cGMP-dependent protein kinase II via interaction with Rab11. Biochem. Biophys. Res. Commun. 374, 522–526
14. Parent, A., Hamelin, E., Germain, P., and Parent, J. L. (2009) Rab11 regulates the recycling of the β2-adrenergic receptor through a direct interaction. Biochem. J. 418, 163–172
15. van de Graaf, S. F., Chang, Q., Mensenkamp, A. R., Hoenderop, J. G., and Bindels, R. J. (2006) Direct interaction with Rab11 targets the epithelial Ca2+ channels TRPV5 and TRPV6 to the plasma membrane. Mol. Cell. Biol. 26, 303–312
16. Hamelin, E., Thériault, C., Laroche, G., and Parent, J. L. (2005) The intracellular trafficking of the G protein-coupled receptor TPβ depends on a direct interaction with Rab11. J. Biol. Chem. 280, 36195–36205
17. Huang, S. H., Wang, J., Sui, W. H., Chen, B., Zhang, X. Y., Yan, J., Geng, Z., and Chen, Z. Y. (2013) BDNF-dependent recycling facilitates TrkB translocation to postsynaptic density during LTP via a Rab11-dependent pathway. J. Neurosci. 33, 9214–9230
18. Büsow, K., Scheich, C., Sievert, V., Hartvig, U., Schulz, J., Simon, B., Bork, P., Lehrahr, H., and Heinemann, U. (2005) Structural genomics of human proteins—target selection and generation of a public catalogue of expres-
32. Schwappacher, R., Rangaswami, H., Su-Yuo, J., Hassad, A., Spitler, R., and Casteel, D. E. (2013) cGMP-dependent protein kinase Iβ regulates breast cancer cell migration and invasion via interaction with the actin/myosin-associated protein caldesmon. J. Cell Sci. 126, 1626–1636
33. Battye, T. G., Kontogiannis, L., Johnson, O., Powell, H. R., and Leslie, A. G. (2011) iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr. D Biol. Crystallogr. 67, 271–281
34. Evans, P. (2006) Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82
35. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221
36. Scapin, S. M., Carneiro, F. R., Alves, A. C., Medrano, F. J., Guimarães, B. G., and Zanchin, N. I. (2006) The crystal structure of the small GTPase Rab11b reveals critical differences relative to the Rab11a isoform. J. Struct. Biol. 154, 260–268
37. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501
38. Winn, M. D., Isupov, M. N., and Murshudov, G. N. (2001) Use of TLS parameters to model anisotropic displacements in macromolecular refinement. Acta Crystallogr. D Biol. Crystallogr. 57, 122–133
39. Bornberg-Bauer, E., Rivals, E., and Vingron, M. (1998) Computational approaches to identify leucine zippers. Nucleic Acids Res. 26, 2740–2746
40. Casteel, D. E., Smith-Nguyen, E. V., Sankaran, B., Roh, S. H., Pilz, R. B., and Kim, C. (2010) A crystal structure of the cyclic GMP-dependent protein kinase Iβ dimerization/docking domain reveals molecular details of isoform-specific anchoring. J. Biol. Chem. 285, 32684–32688
41. Strelkov, S. V., and Burkhard, P. (2002) Analysis of α-helical coiled coils with the program TWISTER reveals a structural mechanism for stutter compensation. J. Struct. Biol. 137, 54–64
42. Pasqualato, S., Sene-Catuglia, F., Renault, L., Goud, B., Salamero, J., and Cherfils, J. (2004) The structural GDP/GTP cycle of Rab11 reveals a novel interface involved in the dynamics of recycling endosomes. J. Biol. Chem. 279, 11480–11488
43. Schlierf, B., Fey, G. H., Hauber, J., Hocke, G. M., and Rosorius, O. (2000) Rab11b is essential for recycling of transferrin to the plasma membrane. Exp. Cell Res. 259, 257–265
44. Schnell, J. R., Zhou, G. P., Zweckstetter, M., Rigby, A. C., and Chou, J. J. (2005) Rapid and accurate structure determination of coiled-coil domains using NMR dipolar couplings: application to cGMP-dependent protein kinase Iα. Protein Sci. 14, 2421–2428