Crystal Structure of G Protein-coupled Receptor Kinase 5 in Complex with a Rationally Designed Inhibitor**

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G protein-coupled receptor kinases (GRKs) regulate cell signaling by initiating the desensitization of active G protein-coupled receptors. The two most widely expressed GRKs (GRK2 and GRK5) play a role in cardiovascular disease and thus represent important targets for the development of novel therapeutic drugs. In the course of a GRK2 structure-based drug design campaign, one inhibitor (CCG215022) exhibited nanomolar IC50 values against both GRK2 and GRK5 and good selectivity against other closely related kinases such as GRK1 and PKA. Treatment of murine cardiomyocytes with CCG215022 resulted in significantly increased contractility at 20-fold lower concentrations than paroxetine, an inhibitor with more modest selectivity for GRK2. A 2.4 Å crystal structure of the GRK5-CCG215022 complex was determined and revealed that the inhibitor binds in the active site similarly to its parent compound GS180736A. As designed, its 2-pyridylmethyl amide side chain occupies the hydrophobic subsite of the active site where it forms three additional hydrogen bonds, including one with the catalytic lysine. The overall conformation of the GRK5 kinase domain is similar to that of a previously determined structure of GRK6 in what is proposed to be its active state, but the C-terminal region of the enzyme adopts a distinct conformation. The kinetic properties of site-directed mutants in this region are consistent with the hypothesis that this novel C-terminal structure is representative of the membrane-bound conformation of the enzyme.

The activation of G protein-coupled receptors (GPCRs)2 via extracellular signals initiates cytoplasmic signaling mediated by heterotrimeric G proteins and their interactions with effector enzymes. However, timely inactivation of these receptors is important for adaptation and minimization of cellular damage and for initiation of alternative signaling pathways. GPCR signaling can be terminated at the level of the receptor by either homologous or heterologous desensitization (1). Whereas heterologous desensitization is mediated by second messenger kinases such as protein kinases A and C (PKA and PKC, respectively), homologous desensitization is mediated by GPCR kinases (GRKs) (2), which selectively phosphorylate agonist-bound GPCRs within either the C-terminal tail (3) or the third intracellular loop (4), creating binding sites for arrestins, which block binding of heterotrimeric G proteins and promote receptor internalization via endocytosis (5).

The seven GRKs (GRK1–7) found in humans can be grouped based on their sequence similarity and gene structure into the GRK1 subfamily (GRK1 and -7), the GRK2 subfamily (GRK2 and -3), and the GRK4 subfamily (GRK4–6) (6). The GRK catalytic core consists of a kinase domain, closely related to that of PKA, inserted into a loop of the regulator of G protein signaling homology (RH) domain (7). However, the most distinguishing characteristic of each subfamily is their C-terminal region, which contains elements that contribute to membrane localization. GRK1 subfamily members have a prenylation site; GRK2 subfamily members have a pleckstrin homology (PH) domain that recognizes both PIP2 and Gβγ subunits (8), and GRK4 subfamily members have a C-terminal amphipathic helix (αCT) that functions as a membrane binding determinant in

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‡The atomic coordinates and structure factors (code 4WNK) have been deposited in the Protein Data Bank (http://wwpdb.org/).

‡‡The abbreviations used are: GPCR, G protein-coupled receptor; AGC kinase, kinase domain related to protein kinases A, G, and C; AST, active-site tether; CaM, calmodulin; GRK, GPCR kinase; Iso, isoproterenol; PH, pleckstrin homology; RH, regulator of G protein signaling homology; ROS, rod outer segment; PDB, Protein Data Bank; AMP-PNP, 5′-adenylyl β,γ-imidodiphosphate.
conjunction with either adjacent palmitoylation sites (GRK4 and GRK6) or with an N-terminal PIP₂-binding site (all members). Studies have shown that whereas the C-terminal helix is sufficient for membrane association (9–11), the N-terminal helix helps the enzyme to adopt a specific orientation at the membrane that may be more optimal for receptor phosphorylation (10).

High resolution crystal structures have previously been reported for representative members of each of the three subfamilies (GRK1, GRK2, and GRK6) revealing the arrangement of their kinase, RH and accessory domains, and providing insight into how they may interact with membrane surfaces and, consequently, GPCRs. For example, GRK2 was crystallized in the complex with Gβγ subunits, and the resulting structure demonstrated how the prenylation site in Gβγ could interact with the same membrane surface as the PIP₂-binding site in the GRK2 PH domain (12). However, there is a conundrum concerning membrane binding by the GRK4 subfamily. The most complete structure of a GRK previously reported, the GRK6-sangivamycin complex (13), revealed the C-terminal membrane-binding amphipathic helix docked in a cleft between its RH and kinase domains, far from the expected membrane binding surface based on the position of the N-terminal PIP₂-binding site and the N-terminal helix, which is anticipated to be a GPCR binding determinant. In addition, it is reported that both the N-terminal region and the amphipathic helix are believed to bind Ca²⁺-CaM (14), which regulates membrane binding and transport of these enzymes (10, 22). Site-directed mutants of residues supporting this C-terminal structure exhibit defects consistent with the idea that the observed packing interactions promote GPCR phosphorylation. The structure therefore represents another high resolution snapshot of a GRK4 subfamily member that provides greater insight into how to direct future rounds of rational drug design against a known cardiovascular target and helps to explain how these enzymes interact with membranes.

**Crystal Structure of the GRK5-CCG215022 Complex**

its close evolutionary relationship to GRK6 permits homology modeling. Most structurally characterized inhibitors have instead been characterized in complex with GRK2, a major target for the treatment of heart failure, or with GRK1, which has been used as a surrogate for GRK5 given their closer homology (6). Herein is described the development of a rationally designed inhibitor, CCG215022, that has nanomolar potency against both GRK2 and GRK5 and is at least 20-fold more potent than paroxetine, as judged by in vitro phosphorylation and cardiomyocyte contractility assays. Based on its ability to strongly protect GRK5 from heat denaturation, it was used to identify crystallization conditions for bovine GRK5. The resulting 2.4 Å structure reveals that the inhibitor engages the so-called hydrophobic subsite of the active site, as do other known potent GRK inhibitors, and that the C terminus of GRK5 adopts a structure distinct from that of GRK6 that is more consistent with the anticipated membrane-bound orientation of these enzymes (10, 22). Site-directed mutants of residues supporting this C-terminal structure exhibit defects consistent with the idea that the observed packing interactions promote GPCR phosphorylation. The structure therefore represents another high resolution snapshot of a GRK4 subfamily member that provides greater insight into how to direct future rounds of rational drug design against a known cardiovascular target and helps to explain how these enzymes interact with membranes.

**Experimental Procedures**

**Protein Purification**—Bovine GRK1(1–535), bovine GRK2 (S670A), wild type and mutant bovine GRK5, and mouse PKA catalytic subunit α were purified via the sequential chromatographic steps of nickel-nitrilotriacetic acid affinity, Source15S, and tandem S200 size exclusion chromatography as described previously (10, 23, 27).

**Inhibition Assays**—GRK kinetic assays were conducted in a buffer containing 20 mM HEPES, pH 7.0, 2 mM MgCl₂, 0.025% n-dodecyl-β-D-maltoside with 50 nM bovine GRK, 5 μM ATP, and 500 nM tubulin in 5-min reactions. Reactions were quenched with SDS loading buffer, separated via SDS-PAGE, dried, and exposed with a phosphorimaging screen prior to quantification via a Typhoon imager. PKA inhibition assays were performed via the ADP-Glo™ system using 0.1 μg of PKA (438 nm), 1 μg of CREBtide substrate, and 100 μM ATP in 30-min reactions. After the initial reaction, ADP-Glo™ reagent was added to the reaction and allowed to incubate for an additional 40 min. Finally the kinase detection reagent was added and incubated for 30 min, and then luminescence was measured with a PHERAstar imaging system. All data were analyzed via GraphPad Prism. For the radiometric GRK data, background-subtracted inhibition curves were fit using a three parameter dose-response curve (fixed slope), wherein the bottom value was constrained to 0. PKA data were not background subtracted and the bottom value was not constrained for each curve.

**Myocyte Shortening Measurements**—Cardiac myocytes were isolated from left ventricular free wall and septum of C57Bl/6 mice as described (24). All cells were used within 2–8 h of isolation. Myocytes were plated on laminin-coated coverslips and were bathed in HEPES-buffered (20 mM, pH 7.4) medium.
199 containing 1.8 mM extracellular Ca\(^{2+}\). When recording, coverslips containing myocytes were mounted in the Dvorak-Stotler chamber and bathed in 0.7 ml of fresh medium. Cells were paced at 1 Hz and imaged with a variable field-rate camera (Zeiss IM35, Ionoptix) by both edge detection and sarcomere length. Peak contraction was measured as the percentage of cell shortening. Cells were treated with isoproterenol (Iso, 0.5 \(\mu\)M) for 2 min for the recording of contraction, with pretreatment of either PBS as vehicle or paroxetine (10 \(\mu\)M) or CCG215022 (0.5 \(\mu\)M) for 10 min (25).

**Crystal Structure Determination—CCG215022 (100 mM stock in DMSO) and MgCl\(_2\) were added to GRK5 to achieve final concentrations of 10 ng/ml GRK5, 1 mM inhibitor, and 5 mM MgCl\(_2\). Crystals were obtained via hanging drop vapor diffusion at 20 °C using a mixture of 0.8 \(\mu\)l of protein (in 20 mM MES, pH 7.0, 50 mM NaCl, 5 mM \(\beta\)-mercaptoethanol, 0.5 mM EDTA, and 1% DMSO) and 0.8 \(\mu\)l of well solution (1 ml), which consisted of 100 mM HEPES, pH 7.5, 4% ethylene glycol, and 12% PEG3350. Crystals appeared in ~1 week and continued to grow in size for at least an additional week. During harvesting, the crystals were cryoprotected by addition of synthetic mother liquor containing 25% ethylene glycol to the drops prior to flash freezing in liquid nitrogen. Diffraction data were collected at the Advanced Photon Source on LS-CAT beamline ID-F at a wavelength of 1.09785 Å. Diffraction data were collected from four total sweeps from a single crystal, which had isotropic diffusion to 2.4 Å spacings. Although the \(I/\sigma\) values suggested useful diffraction extended further, these data were less complete and did not seem to provide additional useful detail to the electron density maps. Indexing, integration, and scaling were performed with HKL2000 (26). A molecular replacement solution was achieved with the PHASER module of CCP4 using PDB entry 4PNK (27) as a search model. Refinement was performed using REFMAC5 (28) alternating with manual fitting in COOT (29). The final model was validated with MolProbity (30) prior to deposition in the PDB as entry 4WNK. Atomic representations were created with the assistance of PyMOL (31).

**Kinetic Analysis of GRK5 Variants—GRK5 and urea-washed bovine rod outer segments (ROS) (32) were mixed in the dark in buffer containing 20 mM HEPES, pH 7.5, 4 mM MgCl\(_2\), and 2 mM EDTA and incubated for 35 min at room temperature. The reaction mixtures were exposed to ambient fluorescent light for 1 min prior to initiation of the reaction by addition of ATP (with \([\gamma^{-32P}]\)ATP) to a final concentration of 1 mM. Final concentration of GRK5 was 100 nM and ROS was between 0.75 and 24 \(\mu\)M. Reactions were initiated at room temperature, and samples were taken at 2–5 min and then quenched with SDS-PAGE loading dye. Proteins were separated using SDS-PAGE, gel was dried, and the incorporation of \(\gamma^{-32P}\) was detected using a phosphor storage screen. Rates at 0 min were plotted against the ROS concentration, and \(V_{\max}\) and \(K_m\) values were determined using the Michaelis-Menten equation. \(V_{\max}\) of each curve was normalized to the \(V_{\max}\) of GRK5561 run in parallel.

**Melting Point Determination—Melting point determinations in response to 200 \(\mu\)M CCG215022 (Table 1) were performed in 20 mM HEPES, pH 7.0, 5 mM MgCl\(_2\), 2 mM DTT, 1 mM CHAPS at a final GRK5 concentration of 0.2 mg/ml and 100 \(\mu\)M anilinonaphthalene-8-sulfonic acid using a ThermoFluor (Johnson & Johnson, New Brunswick, NJ) plate reader. Melting points of GRK5 variants reported in Table 3 were assayed in a buffer containing 20 mM HEPES, pH 8.0, 200 mM NaCl, 2 mM DTT, 2.5

![Crystal Structure of the GRK5-CCG215022 Complex](image-url)
Crystal Structure of the GRK5-CCG215022 Complex

TABLE 1

|           | GRK1 | GRK2 | GRK5 | PKA |
|-----------|------|------|------|-----|
| Balanol   | 0.320e | 0.040e | 0.160e | 0.005e |
| Takeda103A | 0.050e | ND  | ND  | NM  |
| Paroxetine | 230 ± 80 (15)e | 1.6 ± 0.6 (11)e | 480 ± 280 (15)e | ND (3)e |
| GS180736A | 0.5e | 0.77 ± 0.5 (7)e | 250 ± 140 (5)e | 30 ± 20 (5)e |
| CCG215022 | 5.4e | 12.4e | 1.9e | 3.8e |

3 μM ATP, tubulin substrate were used (35).
4 μM ATP, histone substrate were used (51).
500 μM ATP, bovine rod out segment substrate were used (50).
Data were from this study; 5 μM ATP, tubulin substrate were used (25).
Data were from this study; 5 μM ATP, tubulin substrate were used (27).

mM MgCl2 and 0.1 mM anilinonaphthalene-8-sulfonic acid with or without 5 mM ATP. Final GRK5 concentration for these assays was 0.1 mg/ml.

Results

Development of CCG215022—GRK2 and GRK5 are both implicated in the progression of cardiac hypertrophy and heart failure (33, 34), and thus there is interest in the identification of selective chemical probes that could be developed into cardiovascular drugs or be used to deconvolute which of these ubiquitously expressed kinases is involved in desensitizing a specific GPCR in a given cell type or tissue. Recently, paroxetine and a family of indazole-containing compounds were identified in medium-throughput screens as GRK2 inhibitors (25, 27). These compounds all exhibit a similar three-ringed structure that occupies the adenine, ribose, and polyphosphate subunits of the GRK active site (Fig. 1). The most potent of these, GSK180736A, exhibits a 770 nM IC50 value against GRK2 and 300-fold less potency against GRK5. A key structural difference between these compounds and more potent, but less selective, inhibitors such as balanol and Takeda compound 103A (Fig. 1, A and B, respectively) is that they are relatively small and do not occupy the hydrophobic subsite of the kinase-active site (35), thereby exhibiting less total buried surface area (36). Thus, the GSK180736A scaffold was used as a template to design more extended molecules that can occupy the fourth subsite with the goal of enhancing potency while retaining selectivity.3 One such compound, a 2-pyridylmethyl amide derivative (CCG215022), increased the melting point (Tm) of each GRK tested ≥9 °C (Table 1), at least 5 °C greater than that of GSK180736A. Relative to GSK180736A, CCG215022 had 5-fold increased potency of inhibition against GRK2, ≥80-fold against GRK1, and 625-fold against GRK5. As such, the compound is essentially equipotent against GRK2 and GRK5, but it exhibits 10 –20-fold selectivity against GRK1 and 300 –800-fold selectivity against PKA (Fig. 2 and Table 1), although it should be noted that GRK and PKA inhibition is measured using distinct assay formats. Thus CCG215022 serves as a pan-GRK inhibitor that is selective against PKA.

3 H. V. Waldschmidt, K. T. Homan, J. J. G. Tesmer, and S. D. Larsen, manuscript in preparation.

FIGURE 2. Inhibition of GRKs and PKA by CCG215022. Representative curves from an experiment performed in duplicate in the presence of 5 μM ATP. Symbols are as follows: GRK1 (circles), GRK2 (squares), GRK5 (upward triangles), and PKA (downward triangles). See Table 1 (mean IC50 values in the table are slightly different because they represent aggregate measurements).

Potency in Cardiomyocytes—To test whether CCG215022 can inhibit cardiac GRKs and induce increased contractility in a more physiologically relevant context, the response of isolated mouse cardiomyocytes to the compound was measured. At 500 nM in the presence of isoproterenol, CCG215022 generated similar increases in contractility compared with 10 μM paroxetine (Fig. 3). Therefore, CCG215022 was just as efficacious as paroxetine at a 20-fold lower concentration, consistent with the increased potency of inhibition of CCG215022 in vitro versus paroxetine and with GRKs being the principal target of these compounds in murine heart. There was no significant difference with baseline contraction in response to the drug, although the response was generally higher. CCG215022 was also tested at doses of 0.1, 1, and 10 μM (data not shown), but there were no additional effects of 1 and 10 μM versus 0.5 μM after isoproterenol administration.

Crystal Structure of GRK5—Compounds that significantly elevate the Tm of a protein are often good candidates for crystallization. Indeed, CCG215022 allowed for the crystallization of bovine GRK5, ultimately permitting its structure determination to 2.4 Å spacings (Fig. 4A and Table 2). As in most other GRK structures, residues in the extreme N terminus (GRK5 residues 1–23), extreme C terminus (542–590), and active-site tether (AST) region (474–492) are disordered. The
Ast is a feature unique to the PKA, -G, and -C (AGC) kinase family, and it typically becomes more ordered as the kinase domain progresses to an active state (37).

The kinase domain of GRK5 exhibits root mean square deviations of 0.93 (270 C α atoms), 1.4 (252 C α atoms), and 0.69 (268 C α atoms) Å with those of other GRK ligand complexes as follows: GRK1-paroxetine (PDB entry 4L91), GRK2-Gβγ-GSK180736A (4PNK), and GRK6-sangivamycin (3NYN), respectively. The most profound difference among these structures is the relative degree of domain closure. The kinase domain of GRK5-CCG215022 is 10° more closed than GRK2-GSK180736A and 8° more closed than GRK1-paroxetine. Surprisingly, the GRK5 kinase domain exhibits only a 4° difference in domain closure as in the GRK6-sangivamycin complex, wherein the AST and N terminus of the enzyme are ordered and proposed to be in an active configuration. Thus, the difference in domain closure, and/or P-loop conformation in the GRK5-CCG215022 complex, may be sufficient to disrupt these structural elements, which have been proposed to directly interact with GPCRs (13).

Compared with other GRK4 subfamily structures, the GRK5-CCG215022 RH domain exhibits root mean square deviations of 0.6 Å with that of GRK6-AMP-PNP (PDB entry 2ACX), 0.9 Å with that of GRK6-sangivamycin, and 0.75 Å with that of the human GRK5-sangivamycin structure. The GRK6-sangivamycin RH domain is the most structurally divergent among these atomic models, possibly reflecting the fact that its C-terminal region forms unique and extensive interactions at the RH-kinase domain interface, which may induce a distinct conformation in the RH domain.

CCG215022 Interactions—As anticipated, CCG215022 binds to GRK5 similarly to how GSK180736A binds to GRK2 in the GRK2-Gβγ-GSK180736A structure (cf. Fig. 1, A and B) and buries 384 Å² of accessible surface area, 80 Å² more than GSK180736A, which is consistent with other potent GRK inhibitors that occupy the hydrophobic subsite like Takeda101 and Takeda103A (36). The indazole ring binds in the adenine subsite where it forms two hydrogen bonds with backbone atoms in the hinge of GRK5 (Thr-264 and Met-266), and its fluorophenyl ring packs into the polyphosphate subsite, a hydrophobic pocket created between the P-loop and the catalytic Lys-215. However, the dihydropyrimidine and fluorophenyl rings adopt slightly different orientations, and the amide group linking the indazole and dihydropyrimidine rings is flipped between the CCG215022 and GSK180736A structures. These differences are likely due to a greater degree of domain closure in GRK5 relative to GRK2 but also perhaps to the 2-pyridylmethyl amide group introduced into CCG215022. The amide group in this substituent forms two hydrogen bonds with the backbone amide of Phe-197 in the P-loop, displacing Gly-195 and Gly-196 up to 2.6 Å away from the active site, and with the side chain of Asp-329 in the large lobe. The pyridine nitrogen forms a third hydrogen bond with the side chain of Lys-215. Introduction of three new hydrogen bonds relative to GSK180736A may contribute to the pan-GRK inhibition exhibited by this compound (36), especially because all three involve conserved structural elements in the active site. It remains to be seen whether the compound can stabilize an equally closed conformation of GRK2, against which the compound is essentially equipotent. The selectivity against PKA was also observed for the parent GSK180736A compound, and the 2-pyridyl-

4 J. Benovic, personal communication.

**FIGURE 3. Increased adrenergic contractility in myocytes treated with GRK2 inhibitors.** A, representative contraction tracings of single adult ventricular cardiomyocytes showing shortening (%) with a basal twitch and after ISO stimulation. Prior to ISO stimulation, a group of cells was pretreated with paroxetine or CCG215022. B, quantitation of maximal single myocyte contraction amplitude under corresponding conditions. *, p < 0.001 versus baseline; #, p < 0.001 versus DMSO ISO.
methyl amide makes no obvious steric clashes in PKA when modeled into the PKA-balanol complex (38). Thus the selectivity of these compounds against PKA more likely reflects differences in the hinge and overall kinase domain conformation than interactions within the hydrophobic subsite.

**GRK5 C Terminus**—The most prominent structural difference of GRK5 compared with other reported GRK4 subfamily structures, in particular that of the GRK6-sangivamycin complex, is the conformation of its C-terminal tail (residues 527–541) (Fig. 4, A and B). In previously reported crystal structures...
TABLE 2
Crystal refinement statistics

| Protein complex     | GRK5-CCG215022 |
|---------------------|----------------|
| X-ray source        | APS 21 ID-F    |
| Wavelength (Å)      | 1.09785        |
| D_{min} (Å)         | 25.0-2.42 (2.46-2.42) |
| Space group         | P2_1_2_1       |
| Cell constants (Å)  | a = 48.1, b = 70.1, c = 182.8 |
| Unique reflections  | 24217 (1136)   |
| R_{free} (Å)        | 14.1 (100)     |
| Completeness (%)    | 99.9 (100)     |
| (R)/<R>             | 42.3 (4.1)     |
| Redundancy          | 37.7 (34.8)    |
| Refinement resolution (Å) | 25.0-2.42 (2.48-2.42) |
| Total reflections used | 22,906 (1546) |
| r.m.s.d. bond lengths (Å) | 0.011 |
| r.m.s.d. bond angles (%) | 1.483 |
| Estimated coordinate error (Å) | 0.396 |

Ramachandran plot

- Favorable, outliers (%): 96.3, 0.4
- R_{work}: 20.0 (25.8)
- R_{free}: 24.7 (32.1)
- Protein atoms: 4073
- Water molecules: 30
- Inhibitor atoms: 37
- Average B-factor (Å^2): 80.5
- Inhibitor: 53.3
- MolProbity score: 2.44 (100th percentile)
- MolProbity Cβ deviations: 0
- MolProbity bad backbone bonds: 0
- MolProbity bad backbone angles: 0
- PDB code: 4WNK

*Residues in parentheses correspond to the highest resolution shell of data.

In contrast to prior structures of GRK6 and GRK1, the C terminus of GRK5 adopts a completely different course (Fig. 4, A and B), although one that is consistent with the trajectory indicated by the final residues observed in the GRK1-L166K structure (residues 531–532). The GRK5 C-terminal region first forms a short loop (residues 527–533), the ends of which were anchored by side chain packing interactions of Phe-527 and Leu-533 with that of Met-165 in the RH domain (analogous to Leu-166 in GRK1). All three residues are highly conserved among GRK5 and GRK6 enzymes (Fig. 4C). The loop is followed by a single α-helical turn (residues 534–437) that contains an invariant PDL sequence signature. Pro-535 caps the beginning of the helix; Asp-536 forms a salt bridge with Arg-169 and a hydrogen bond with Asn-172 in the RH domain, and Leu-537 serves as a third hydrophobic anchor with its side chain packing between Ile-39 and Met-165 from the RH domain. The remainder of the observed C-tail wraps around the side chains of His-38 and Ile-39; finally Arg-539, conserved only in GRK5, forms a salt bridge with Asp-168 in the RH domain. Thus, the tail sequesters much of the region involved in the molecular 2-fold related interfaces observed in previous GRK6 and GRK1 structures. Sequence analysis suggests that GRK1 and GRK6 should be able to form a similar C-terminal structure (Fig. 4C), but other sequence differences make it difficult to predict whether GRK4 will form an analogous C-terminal structure. The αCT helix, thought to regulate interactions with the phospholipid membrane (9, 42), begins a few residues after the last observed residue of GRK5. However, the helix would now be positioned so that it could be in the same plane as the phospholipid-binding sites near the N terminus of the enzyme (Fig. 6).

Functional Analysis of the C Terminus—To test the role of the C-terminal structure of GRK5, two classes of site-directed mutants in GRK5 were created. All were made in the background of bovine GRK5 truncated after residue 561, which corresponds to the last residue observed in the GRK6-sangivamycin structure and should eliminate any effects from the rest of the C terminus, which could play additional regulatory roles (43). The first set, consisting of R64A, A88K/E89K, and P546A, was predicted to disrupt packing if it was similar to the GRK6-sangivamycin C-terminal structure (Fig. 4A). In the second set, the D536A and L537A variants were predicted to disrupt the packing of both. If the GRK6-sangivamycin structure represents an
autoinhibited and/or soluble conformation that GRK5 can also attain, then mutations in the first class would be expected to increase catalytic activity because the C-terminal amphipathic helix would be more free to interact productively with the membrane. If the GRK5/CCG215022 structure represents a more active conformation, then mutations in the second class could be inhibitory. As shown in Table 3, both D536A and L537A variants exhibited significantly diminished $V_{\text{max}}$ values, whereas the remaining mutations either elevated the catalytic rate (R64A and A88K/E89K) or had no effect (P546A). The ability of these mutants to alter the melting point of GRK5 was also evaluated. All variants except L537A and P546A significantly decreased the melting point of the ligand-free enzyme. Despite this, all demonstrated a similar increase in $T_m$ upon addition of the substrate 5 mM ATP, suggesting that they were properly folded. The $T_m$ data are thus consistent with a model wherein the C terminus of GRK5 can switch between GRK6-sangivamycin-like and GRK5/CCG215022-like structures, with the former being more resistant to denaturation, as might be expected from the larger amount of buried surface area and sequestration of the $\alpha$CT helix (Fig. 6).

**Discussion**

The CCG215022 inhibitor was designed based on the hypothesis that if the hydrophobic subsite of the kinase domain was occupied, as it is by inhibitors such as balanol and the Takeda compounds (Fig. 1), then there would be a corresponding increase in potency. The GRK5/CCG215022 structure validated this design principle because the 2-pyridylmethyl adduct occupies this subsite, burying an additional 80 Å² of surface area relative to the parent compound (albeit bound to GRK2) (27) and exhibited almost 3 logs of improved potency against GRK5 from the parent compound GSK180736A. CCG215022 also exhibited at least 20-fold better potency in a cardiomyocyte contractility assay than paroxetine (Fig. 3). However, CCG215022 exhibited only a relatively small improvement in the potency of inhibition against GRK2 despite being able to increase the $T_m$ of all tested GRKs (Table 1). The reasons for this are not clear, but it could reflect the fact that the hydrophobic subsite of GRK2 is larger than that of GRK5 and is less able to complement the relatively small pyridine ring of CCG215022. Indeed, the terminal ring structures of the Takeda compounds, which are highly selective for GRK2, are bulkier difluorobenzyl and trifluoromethylbenzyl groups (Fig. 5, B and C). Exploiting this size difference in the hydrophobic subsite is therefore a promising route for the design of more selective GRK2 inhibitors.

CCG215022 stabilizes a relatively closed conformation of the GRK5 kinase domain most similar to that exhibited by the kinase domain in the GRK6-sangivamycin structure, which in turn has been proposed to be the most similar to an active GRK. However, unlike the GRK6-sangivamycin complex, the N terminus and AST regions of GRK5 are not ordered, suggesting an inactive state. There are several possible explanations for this discrepancy. The first is that the CCG215022 inhibitor perturbs the P-loop of the active site such that the N-terminal helix cannot stably interact with the small lobe or the AST. Alternatively,
formation of a stable N-terminal helix/AST interaction may be highly dependent on intermolecular contacts, which in the GRK6-sangivamycin structure were provided by crystal lattice interaction, and perhaps in cells by activated GPCRs. The GRK6-AMP-PNP structure (39) exhibits a relatively open kinase domain conformation that would also preclude its ability to form the same N-terminal helix/AST structure as observed in GRK6-sangivamycin.

Another question that emerges from the GRK5-CCG215022 structure is the dramatic difference in the structure of its C terminus from that observed in the GRK6-sangivamycin and GRK6-AMP-PNP complexes, even though GRK5 and GRK6 are very close homologs. In the GRK6-sangivamycin and GRK6-AMP-PNP structures, a crystallographic dimer drives a domain swap that would prevent the C terminus from forming the same structure as observed in GRK5-CCG215022. The C terminus of the GRK6-sangivamycin structure is better ordered than in GRK6-AMP-PNP, perhaps due to differences in their kinase domain conformations, which in turn alter interactions between the RH and kinase domains that form a docking site for αCT (Fig. 4A). However, the C terminus of GRK5-CCG215022 is also involved in a crystal contact, which may stabilize an artifactual conformation. Fortunately, prior to this structure determination, the Benovic lab determined crystal structures of human GRK5 co-crystallized with sangivamycin (as in GRK6-sangivamycin) and the nonhydrolysable ATP analog AMP-PNP (see accompanying article (52)). Superposition of the RH domains of these two structures demonstrates that despite different lattice packing constraints, they form very similar C-terminal structures, with the most prominent difference being the conformation of the loop that begins the C-terminal tail. In the human GRK5 structures, this region is free of lattice contacts but exhibits relatively high temperature factors. Thus, the C-terminal structure observed in these three GRK5 models likely represents a relevant conformational state, and one that would place the C-terminal αCT helix of GRK5 (albeit disordered) in position to interact with the same membrane plane as residues near the N terminus that bind to PIP2 (Fig. 6) (22).

The kinase domains in the human GRK5 structures (52) adopt an intermediate degree of domain closure compared with GRK6-sangivamycin, which is the most similar to the transition state conformation of PKA (44), and human GRK6-AMP-PNP, which is the most open. Thus, a specific kinase domain conformation is not necessarily achieved by co-crystallization with a specific nucleotide analog at least in the GRK4 subfamily. Rather, the kinase domain conformation, and those of its N terminus and AST region, seems strongly influenced by lattice contacts. This conformational plasticity may underlie the ability of these enzymes to be activated by a wide range of GPCRs. Another intriguing difference between the human GRK5 structures reported by Komolov et al. (52) and the GRK5-CCG215022 complex is the fact that the AST region of the human structure is well ordered. Stabilization of the AST is a hallmark of activation in the AGC family of kinases, and the human GRK5 structures were indeed determined in complex with ATP analogs, suggesting that they may reflect an active form of the enzyme. However, the human GRK5 AST does not follow a typical trajectory as observed in prior AGC kinase (37) and GRK structures (13, 40), and catalytic residues in the active site are not aligned for phosphotransfer. Notably, noncanonical conformations of the AST have been observed in other GRK structures, such as in the GRK1-GSK2163632A (27) and GRK6-AMP-PNP (39) complexes. Indeed, the hydrophobic side chains of Tyr-473, Val-489, and Leu-491 in the human GRK5 AST are all involved in strong lattice contacts. It thus remains to be determined what the structural difference in the AST region represents in terms of the physiological function of GRK5.
Crystal Structure of the GRK5-CGG215022 Complex

TABLE 3
Functional analysis of the GRK5 C-terminal structure

|                  | V_mmax (µM) | K_m | V_mmax/K_m | ΔT_m | ΔT_m + ATP |
|------------------|-------------|-----|------------|-------|------------|
| GRK5561          | 97 ± 5      | 3.5 ± 0.1 | 28       | 0     | 7.7 ± 0.3  |
| GRK5561 R64A     | 193 ± 26    | 4.9 ± 1.8 | 39       | -1.3 ± 0.1***  | 5.5 ± 0.6* |
| GRK5561 A88K/89K | 102 ± 13    | 2.5 ± 1.1 | 41       | -0.9 ± 0.1**   | 6.6 ± 0.2  |
| GRK5561         | 58 ± 2.9    | 3.7 ± 1.7 | 16       | -0.9 ± 0.2**   | 6.6 ± 0.4  |
| GRK5561 L537A    | 61 ± 7.7    | 4.3 ± 1.5 | 14       | -0.4 ± 0.2     | 6.5 ± 0.4  |
| GRK5561 F546A    | 136 ± 16    | 4.6 ± 1.5 | 30       | 0.2 ± 0.1      | 7.9 ± 0.3  |

It also remains to be determined whether the C-terminal structure exhibited by the GRK6-sangivamycin complex is physiologically relevant. The activity and T_m values presented in Table 3 are consistent with the C terminus of GRK5 being able to adopt a GRK6-sangivamycin-like conformation, which is predicted to stabilize the overall fold of the enzyme and increase the melting point. Mutations that disrupt this structure would allow for more kinase domain flexibility, lower melting point, and increased activity. Thus, the GRK6-sangivamycin-like conformation may reflect a soluble form of GRK5/6 that shields the C-terminal helix from nonspecific interactions, akin to the myristoyl switch found in other proteins whose functions require transient interaction with the membrane (45, 46). In addition, it has also been noted by multiple labs that GRK6 can have orders of magnitude less activity than other GRKs, including GRK5, on the same receptor substrate (23, 47), yet GRK6 retains similar activity against soluble substrates (47). Therefore, the C terminus of GRK6 may have a greater propensity to form a GRK6-sangivamycin-like conformation than GRK5. A possible explanation for this phenomenon could be that GRK6-TRp-539, an insertion in the C terminus relative to GRK5 (Fig. 4C), makes a key anchoring contact in the GRK6-sangivamycin structure that may influence conformational equilibrium (Fig. 6). If so, the C terminus as observed in the GRK6-sangivamycin structure may help explain why GRK6 is not constitutively associated with membranes, whereas GRK5 is (48).

Finally, the three new structures of GRK5 also help to clarify how Ca^{2+}•CaM mediates inhibition of GRK4 subfamily members. Ca^{2+}•CaM has been reported binding sites within GRK5 at both the N terminus, just after the N-terminal helix (residues 20–39) (49), and at the C terminus (residues 545–560), which encompasses the C-terminal amphipathic helix (14). If the GRK5-CGG215022 structure indeed represents a membrane-bound conformation, then these two sites are now in much closer proximity than previously recognized. It is possible that Ca^{2+}•CaM can bind to both sites simultaneously, as suggested by multilength light scattering analysis of their complex, which indicates 1:1 stoichiometry (data not shown). Thus, the C termini of the GRK4 subfamily members may play more complex regulatory roles than previously thought by serving as phosphorylation sites, protein-binding sites, conformational switches, and membrane targeting modules.

Author Contributions—K. T. H. and J. J. G. T. conceived and coordinated the study and wrote the paper. H. V. W. and S. D. L. designed and synthesized the CCG215022 inhibitor. K. T. H. purified GRK5 and crystallized and solved the structure of the GRK5-CGG215022 complex; both K. T. H. and J. J. G. T. built and refined the atomic model. K. T. H. performed the inhibition studies shown in Fig. 2 and Table 1. A. C., J. S., J. Y. C., and W. J. K. performed the contractility experiments described in Fig. 3. A. G. was responsible for creating, purifying, and assay GRK5 mutations as shown in Table 3. All authors reviewed the results and approved the final version of the manuscript.

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