

Structural insight into guanylyl cyclase receptor hijacking of the kinase–Hsp90 regulatory mechanism

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Abstract

Membrane receptor guanylyl cyclases play a role in many important facets of human physiology, ranging from regulation of blood pressure to the regulation of intestinal fluid secretion. The structural mechanisms which influence the regulation of these important physiological effects have yet to be explored. We present the 3.9 Å resolution cryoEM structure of the human membrane receptor guanylyl cyclase GC-C in complex with Hsp90 and its co-chaperone Cdc37, providing insight into the mechanism of Cdc37 mediated binding of GC-C to the Hsp90 regulatory complex. As a membrane protein and non-kinase client of Hsp90–Cdc37, this work shows remarkable plasticity of Cdc37 to interact with a broad array of clients having significant sequence variation. Further, this work shows how membrane receptor guanylyl cyclases hijack the regulatory mechanisms used for active kinases to facilitate their regulation. Given the known druggability of Hsp90, these insights can guide the further development of mGC targeted therapeutics and lead to new avenues to treat hypertension, inflammatory bowel disease, and other mGC related conditions.

eLife assessment

Caveney et al. overexpressed an engineered construct of the human membrane receptor guanyl cyclase GC-C in Hamster cells, co-purified it with endogenous HSP90 and CDC37 proteins and determined the cryo-EM structure of this complex. This **important** work shows that the pseudo-kinase domain of GC-C associates with CDC37 and HSP90 similarly to how the bona fide protein kinases CDK4, CRAF and BRAF have been shown to interact. The methodology used is state of the art and the evidence presented is **convincing**.

Introduction

Cyclic GMP (cGMP) is an important second messenger for signaling in mammalian physiology, with roles in platelet aggregation, neurotransmission, sexual arousal, gut

peristalsis, bone growth, intestinal fluid secretion, lipolysis, phototransduction, cardiac hypertrophy, oocyte maturation, and blood pressure regulation (Potter, 2011). Largely, cGMP is produced in response to stimuli by guanylyl cyclases (GC), a class of receptors which contains both heteromeric soluble receptors (α_1 , α_2 , β_1 and β_2 in humans) and five homomeric membrane receptors (GC-A, GC-B, GC-C, GC-E, GC-F in humans). Of note are the membrane receptor guanylyl cyclases (mGC) GC-A and GC-B, also known as natriuretic peptide receptor A and B (NPR-A and NPR-B), respectively, and GC-C, all of which have been a focus of therapeutic development. In the case of NPR-A and B, their role in the regulation of blood pressure in response to natriuretic peptide hormones (ANP, BNP, CNP) has led to the exploration of agonists for use in the treatment of cardiac failure (Kobayashi et al., 2012). Meanwhile, GC-C is the target of clinically approved laxative agonists, linacotide and plecanatide (Miner, 2020; Yu & Rao, 2014), due to its role in the regulation of intestinal fluid secretion.

These membrane receptor GCs consist of an extracellular ligand binding domain (ECD), which acts as a conformational switch to drive intracellular rearrangements to activate the receptor (X. L. (He et al., 2001a)); a transmembrane region (TM); a kinase homology domain or pseudokinase domain (PK); a dimerization domain; and a guanylyl cyclase (GC) domain, which acts to produce cGMP. The PK domain is largely thought to be involved in scaffolding and physical transduction of the extracellular rearrangements to the GC domain, in some respects similar to the role of the PK domain in the Janus kinases of the cytokine signaling system (Glassman et al., 2022). In addition, the PK domains of mGCs are regulated through their phosphorylation state (Potter & Garbers, 1992; Potter & Hunter, 1998; Vaandrager et al., 1993) and via association with heat shock proteins (Hsp) (Kumar et al., 2001).

While the role of phosphorylation state on mGC activity has been explored in relative detail, how the heat shock protein 90 (Hsp90) is able to regulate mGC activity is largely unknown. It has been shown that GC-A activity can be regulated through the association of Hsp90 and the co-chaperone Cdc37 (Kumar et al., 2001). The chaperone Cdc37 is known to assist in the Hsp90 regulation of around 60% of active kinases, both in soluble and membrane receptor form (Taipale et al., 2012). Given the sequence and structural similarities between the PK domains of mGCs and the active kinase domains which are regulated by Hsp90–Cdc37, it is possible that mGCs have evolved to hijack the regulatory mechanisms that are more broadly deployed for active kinases.

Here we report the 3.9 Å resolution structure of the GC-C–Hsp90–Cdc37 regulatory complex. In this structure, the core dimer of Hsp90 forms its canonical closed conformation, while Cdc37 and the C-lobe of the GC-C PK domain asymmetrically decorate the complex. The client (GC-C) is unfolded into the channel formed at the interface between the Hsp90 dimers. To our knowledge, this is the first structure of a membrane protein client of Hsp90 and the first structure of a non-kinase client of the Hsp90–Cdc37 regulatory system. This work provides pivotal understanding of the mechanism and structural basis of kinase fold recruitment to the Hsp90–Cdc37 regulatory complex. This increased understanding can guide the further development of mGC targeted therapeutics and lead to new avenues to treat hypertension, inflammatory bowel disease (IBD), and other mGC related conditions. In addition, the general insights into recruitment of Hsp90–Cdc37 clients can guide the further development of Hsp90 targeting therapeutics in cancer treatment.

Results

Structure of the GC-C-Hsp90-Cdc37 regulatory complex

Membrane receptor guanylyl cyclases have been largely recalcitrant to structural analysis by x-ray crystallography and electron microscopy, apart from various crystal structures of both liganded and unliganded ECDs (X. L. (He et al., 2001b); X. lin (He et al., 2006); (Ogawa et al., 2004), (2010); (van den Akker et al., 2000)). Given the relative disparity between our structural understanding of the ligand binding extracellular domains and that of the functional intracellular domains, we sought to develop a stable construct to image and gain crucial understanding of the regulatory and functional aspects of mGCs which occur intracellularly. By replacing the ligand-responsive ECD with a homodimeric leucine zipper, we mimic the ligand activated geometry of the ECD (X. L. (He et al., 2001b)), while reducing complexity of the imaged complex and increasing stability (Figure 1A). This complex was recombinantly expressed in mammalian cell culture, purified with affinity chromatography, and vitrified on grids for cryoEM analysis.

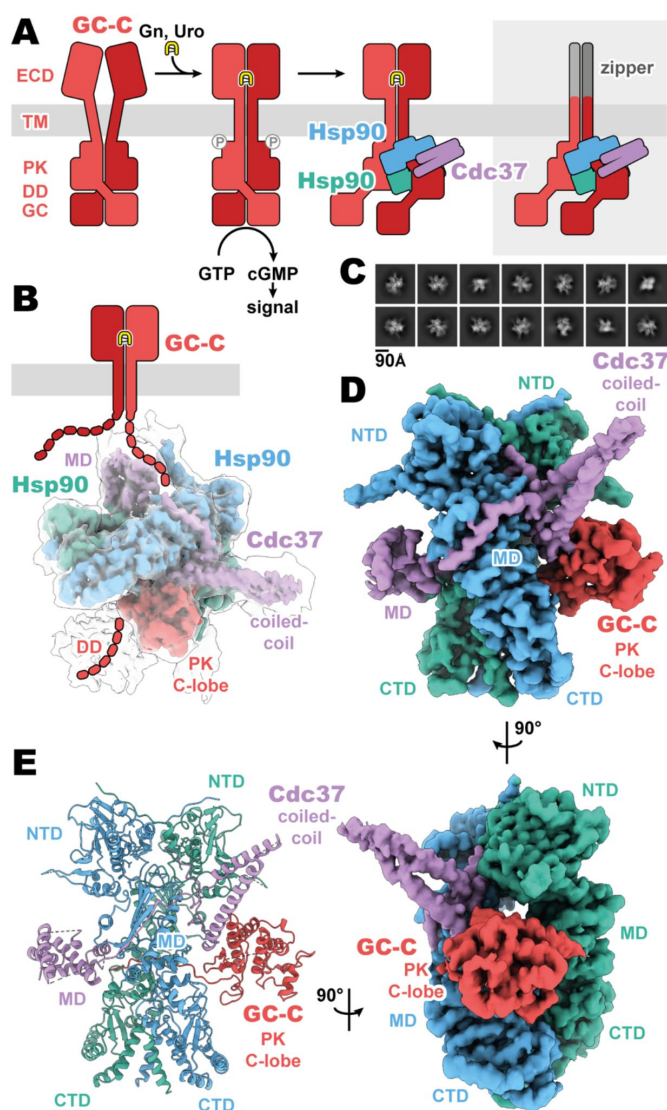


Figure 1.

Composition and cryoEM structure of the GC-C-Hsp90-Cdc37 regulatory complex.

(A) Cartoon representation of the components of GC-C signaling and Hsp90-Cdc37 regulation and the zippered and activated GC-C. GC-C is colored in red, guanylin/uroguanylin (Gn/Uro) in yellow, Hsp90 in blue and teal, and Cdc37 in purple. Extracellular domains (ECD), transmembrane domain (TM), pseudokinase domain (PK), dimerization domain (DD), and guanylyl cyclase domain (GC) are labelled. (B) The refined and sharpened cryoEM density map of GC-C-Hsp90-Cdc37, colored as in A, with a transparent overlay of an unsharpened map with additional DD density resolved. Cdc37 coil-coiled and middle domain (MD) are labelled. (C) Reference free 2D averages for the GC-C-Hsp90-Cdc37 complex. (D) The refined and sharpened cryoEM density map of GC-C-Hsp90-Cdc37, colored as in A and B, labelled with all domains as in A and B, with the addition of Hsp90 N-terminal domain (NTD), middle domain (MD), and C-terminal domain (CTD). (E) Ribbon representation of a model of GC-C-Hsp90-Cdc37 complex, colored and labelled as in A, B, and C.

The purified sample had a significant portion of imaged particles for which the native regulatory heat shock protein, Hsp90, and its co-chaperone, Cdc37, are bound. The *Cricetulus griseus* HSP90 β and Cdc37 show remarkable sequence conservation in comparison to the human equivalents, at 99.7 and 94.2% identity, respectively. This native pulldown strategy contrasts with the structures of Hsp90–Cdc37 in complex with soluble kinases (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016), for which Hsp90 and Cdc37 had to be overexpressed to obtain complex suitable for imaging. Three-dimensional reconstruction of our GC-C–Hsp90–Cdc37 particles generated a 3.9 Å resolution map of the regulatory complex (Figure 1, Supplementary Figure 1). A second, unsharpened, map from subsequent heterogenous refinement resolves additional density for dimerization domain, extending outward from the PK domain (Figure 1B, Supplementary Figure 1).

The resultant GC-C–Hsp90–Cdc37 complex is a hetero-tetramer formed by one resolved monomer of the GC-C receptor bound to a dimer of Hsp90 and one Cdc37 co-chaperone (Figure 1D). As observed with most Hsp90–client structures, the bulk of the complex is composed of the C2 pseudosymmetric closed state Hsp90 dimer. Building on this dimeric core, the Cdc37 protrudes outward from one side with its characteristic long, coiled-coil, α -hairpin. On one face of the Hsp90 dimer core, Cdc37 interacts with the PK domain of GC-C, while an extended β -sheet wraps around to the other face, lying across and extending a β -sheet in the middle domain (MD^{Hsp90}) of one Hsp90 monomer. At the opposite face, the globular and α -helical Cdc37 middle domain (MD^{Cdc37}) is formed. The C-lobe of the GC-C PK domain packs against the N-terminal region of Cdc37 on one face of the dimeric Hsp90 core, with the N-lobe unfolding through the dimer core to interface with the MD^{Cdc37} on the opposite face. N-terminal to the PK N-lobe is the TM region, density for which was unobserved in our reconstructions. C-terminal to the PK C-lobe, we observe some poorly resolved density for the likely mobile dimerization domain in our unsharpened map. This would precede the GC domain, which is not observed in the density of our reconstructions (Figure 1B). Together, we can use our understanding of mGC topology and our reconstruction to orient the complex as it would sit on a membrane (Figure 1B), providing insight into how Hsp90 is able to access and regulate membrane protein clients. No density is observed for the second GC-C of the dimer, though it is sterically unlikely that an additional regulatory complex is forming on the second GC-C in a concurrent fashion.

Cdc37 mediated GC-C recruitment and Hsp90 loading

Given the remarkable plasticity of Cdc37 co-chaperone binding to 60% of kinases (Taipale et al., 2012) and an unknown number of pseudokinase domain containing proteins in the human proteome, the structural basis for how Cdc37 can recruit GC-C to the Hsp90 regulatory complex is of particular interest. In our structures, we see that Cdc37 is displacing the N-lobe of the pseudokinase domain of GC-C, binding to the C-lobe at the N–C interface, and guiding the unfolded N-lobe into the Hsp90 dimer (Figure 2). The Cdc37–GC-C interface is relatively modest in size, with a calculated mean surface area of 689 Å² (as calculated by PISA (Krissinel & Henrick, 2007)). This interface is in part driven to form via charge complementarity, with positive contributions from a cluster of arginine residues on Cdc37 (R30, 32, 39) at the periphery of the interaction interface interacting with D609 and the polar residues Y580 and T586 (Figure 2B). Beyond this, the interface is likely largely driven via shape-complementarity, due to a minimal contribution from hydrogen bonding, salt-bridge formation, and aromatic packing contributions – in line with the ability of Cdc37 to chaperone such a diverse array of clients and client sequences.

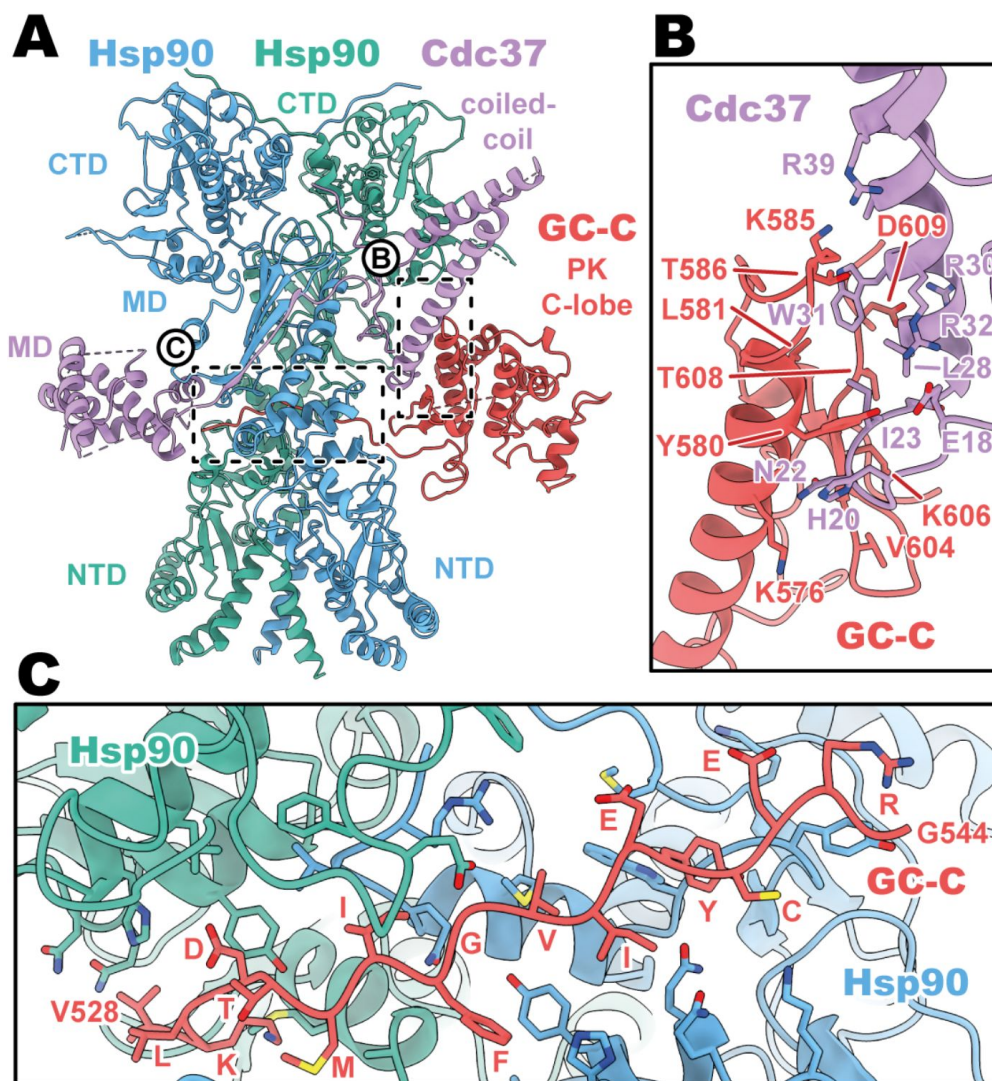


Figure 2.

Cdc37 mediated GC-C recruitment and Hsp90 loading interfaces.

(A) Ribbon representation of a model of GC-C-Hsp90-Cdc37 complex. GC-C is colored in red, Hsp90 in blue and teal, and Cdc37 in purple. Pseudokinase (PK), coil-coiled, middle (MD), C-terminal (CTD), and N-terminal (NTD) domains are labelled. (B) The Cdc37-GC-C interface in ribbon representation, with interacting residues drawn in sticks, colored as in A. (C) The unfolded N-lobe of GC-C PK domain as it passes between the Hsp90 dimer, in ribbon representation, with interacting residues drawn in sticks, colored as in A and B. This region's sequence is: VKLDTMIFGVIEYCERG.

As the unfolded PK N-lobe extends away from Cdc37, it enters the channel formed at the interface between the dimer of Hsp90 (Figure 2C). Here, GC-C residues 528 to 544 (VKLDTMIFGVIEYCERG) lie across the upper region of the Hsp90 CTDs, which form the floor of the channel. These CTDs form the bulk of the interaction interface as the unfolded N-lobe passes through this channel, yet there are minor contributions from the loop regions of the β -sheet from the MD^{Hsp90} which extend downward into this channel region. The unfolded region is relatively poorly resolved in the density, with some reconstructions from earlier refinement having no resolvable density in this channel region (not shown) – indicative of the low stability and high mobility for the unfolded N-lobe as it passes through this region.

Conservation of Cdc37 mediated Hsp90 regulation

The core structural principles of Cdc37 mediated client recruitment to Hsp90 appear to remain constant across its large range of client diversity. Across other client-Hsp90-Cdc37 complexes with canonical soluble kinase clients (Cdk4, RAF1, B-raf) (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016), we see a conserved role for Cdc37 in client recruitment by associating with the C-lobe at the N-, C-lobe interface (Supplemental Figure 2A). In these complexes we see high levels of structural conservation for the Hsp90-Cdc37 (Ca RMSDs of 1.4-3.3 Å for Hsp90 and 1.5-2.5 Å for Cdc37), while the client is structurally

most homogenous at the interface with Cdc37, though less structurally conserved overall (Ca RMSDs of 3.5-11.6 Å). Perhaps unsurprisingly, GC-C is one of the most divergent of these clients from a sequence perspective (Supplemental Figure 2B), with sequence homology between the GC-C PK domain and the other client kinase domains ranging from 19 to 25% identity and 31 to 41% homology. This highlights the plasticity required of this system which can service such a vast array of clients across a broad range of sequence variation, yet more restricted fold architecture.

Discussion

The present cryoEM structure of GC-C–Hsp90–Cdc37 resolves the loading of GC-C, via its PK domain and interaction with Cdc37, to the Hsp90 core dimer (Figure 1, 2). This complex shows significant structural similarity to the mechanism used to regulate soluble active kinases (García-Alonso et al., 2022; Oberoi et al., 2022; Verba et al., 2016) and presumably membrane receptor kinases in the human proteome. This structural and mechanistic conservation is largely driven by the co-chaperone Cdc37, which serves as the main binding platform for these clients by associating to the fold of the kinase (or pseudokinase in the case of mGC) domain, relatively independent of sequence identity. A model whereby recruitment is largely driven by both the fold complementarity and the specific stability properties of the kinase fold has been proposed previously (Taipale et al., 2012). In this model, instability of a fully folded kinase domain results in partial unfolding of the C-lobe, leading Cdc37 to bind the partially unfolded state. Given the lack of functional and sequence conservation for GC-C as a client of Cdc37, our data largely fits with this model for client recruitment. It is likely that the pseudokinase domains of mGC have largely evolved to facilitate regulatory mechanisms for these receptors, both via their phosphorylation and by hijacking the regulatory mechanisms used by active soluble and membrane receptor kinases.

In the case of GC-A, previous work has shown that it associates with the Hsp90–Cdc37 complex to regulate GC activity (Kumar et al., 2001). The authors showed both that the addition of geldanamycin, an Hsp90 inhibitor, reduces the overall cGMP output of cells in response to ANP stimulation while also reducing the association of the Hsp90 to GC-A. While this initially may seem counterintuitive, this data fits with a model of ligand-induced activity potentiating the instability of the PK domain, which then facilitates binding of the regulatory complex to “re-fold” GC-A for further catalysis and cGMP production – in a core regulatory complex structurally similar to that which we observe for GC-C in this work (Supplemental Figure 3). In the case of the Hsp90 inhibitor, this would release the Hsp90 and only allow full catalytic activity for the receptor until the receptor falls into the partially unfolded state, as the Hsp90 would no longer be able to re-engage at the C-lobe when inhibited (Supplemental Figure 3).

Interestingly there may be an additional layer of regulation involved, with crosstalk between the phosphorylation and Hsp90 regulatory mechanisms of mGC. The phosphatase PP5 is known to interact with the Hsp90–Cdc37 system and dephosphorylate Hsp90, Cdc37, and the system’s kinase clients (Oberoi et al., 2022). PP5 has been implicated in this role for mGC (Chinkers, 1994), though this interaction was unable to be detected by a pull-down in a second study (Kumar et al., 2001). In this way, mGC association to the Hsp90–Cdc37 could result in multiple fates and resultant activity profiles for the receptor. When the PK of an activated mGC falls into a destabilized state, this would result in the recruitment of the Hsp90–Cdc37. First the regulatory complex could refold the receptor to maintain activity of the receptor (Supplemental Figure 3 i). In another scenario, the Hsp90–Cdc37 complex could additionally recruit PP5 to dephosphorylate the mGC (Supplemental Figure 3 ii). Particularly in the case of GC-A and GC-B, and to some extent GC-C (Potter & Garbers, 1992; Potter &

Hunter, 1998; Vaandrager et al., 1993), this would impair the signaling activity of the mGC, though this could be rescued through the kinase re-association and phosphorylation. In a final scenario, the binding of the Hsp90–Cdc37 complex could result in the association of ubiquitin E3 ligases (Schopf et al., 2017) (Supplemental Figure 3 iii), which would ubiquitinate the mGC client, leading to the removal of the receptor.

The regulation of mGC is influenced by a network of factors working in harmony to ensure proper signaling and physiological response for these important receptors. The structure of the core regulatory complex shown in this work is key to many facets of mGC regulation. We hope that the structural basis for the Hsp90 regulatory platform for mGC will drive renewed investigation in these diverse mechanisms and lead to the therapeutic manipulation of these mechanisms to improve mGC targeting therapies.

Methods

Cloning and protein expression

For cryoEM studies, a construct containing an HA secretion signal (MKTIALSYIFCLVFA), a FLAG peptide (DYKDDDD), linker and 3C cleavage site (KGSLEVLFGPG), GCN4 homodimeric zipper (RMKQLEDKVEELLSKNYHLENEVARLKKLVGER), human GC-C regions corresponding to the small extracellular linker region, TM, and GC domain (residues 399-1,053), a second linker and 3C cleavage site (AAALEVLFGPGAA), a Protein C epitope tag (EDQVDPRLIDGK), and an 8x His tag were cloned into a pD649 mammalian expression vector. Protein was expressed in ExpiCHO cells (GIBCO) maintained in ExpiCHO Expression Media (GIBCO) at 37°C with 5% CO₂ and gentle agitation. Cells were pelleted and stored at -80°C.

Protein purification

Cells were resuspended in 20 mM HEPES pH 8.0, 300 mM NaCl, 1 mM TCEP, protease inhibitor cocktail (Sigma), and benzonase (Sigma). Cells were lysed by Dounce homogenization and cellular debris were pelleted by low-speed centrifugation at 500 x g. Membranes were collected by ultracentrifugation at 46,000 x g and stored at -80°C until use. Membranes were thawed and solubilized with the addition of 1% n-dodecyl β -D-maltoside (DDM) and 0.1% cholesteryl hemisuccinate (CHS) (10:1) (Anatrace). Debris and unsolubilized membranes were pelleted by ultracentrifugation at 46,000 x g. The supernatant was subsequently used in FLAG affinity chromatography. The supernatant was applied to M1 antiFLAG resin. The resin was washed with 20 bed volumes of 20 mM HEPES pH 8.0, 300 mM NaCl, 1 mM TCEP, 0.005% lauryl maltose neopentyl glycol LMNG, 0.0005% CHS (10:1) (anatrace), and 5 mM ATP. The protein complex was eluted with the addition of 200 μ g/mL of FLAG peptide (DYKDDDD) (GenScript). Protein was subsequently concentrated to >2 mg/mL and used for cryoEM imaging.

Cryo-electron microscopy

Aliquots of 3 μ L of complex were applied to glow-discharged 300 mesh UltrAuFoil® (1.2/1.3) grids. The grids were blotted for 3 seconds at 100% humidity with an offset of 3 and plunge frozen into liquid ethane using a Vitrobot Mark IV (Thermo Fisher). Grid screening and dataset collection occurred at Stanford cEMc on a 200 keV Glacios microscope (Thermo Fisher) equipped with a K3 camera (Gatan). Movies were collected at a magnification corresponding to a 0.9273 Å per physical pixel. The dose was set to a total of 58.8 electrons

per Å². Automated data collection was carried out using SerialEM with a nominal defocus range set from -0.8 to -2.0 µM.

Image processing

All processing was performed in cryoSPARC (Punjani et al., 2017) unless otherwise noted (Supplementary Figure 1). 8,788 movies were motion corrected using patch motion correction. The contrast transfer functions (CTFs) of the flattened micrographs were determined using patch CTF and an initial stack of particles was picked using Topaz picker (Bepler et al., 2019). Successive rounds of reference-free 2D classification were performed to generate a particle stack of 165,635 particles. These particles were then used in ab-initio reconstruction, followed by non-uniform refinement (Punjani et al., 2020) and finally local refinement with a loose mask around the entire complex. This resulted in a 3.9 Å reconstruction of the GC-C–Hsp90–Cdc37 complex. These particles were also used in a 4 class heterogenous refinement to pull out a volume containing some resolved density for the dimerization domain of GC-C.

Model building and refinement

The Cdk4–Hsp90β–Cdc37 (PDB 5FWK), PP5–B-Raf–Hsp90β–Cdc37 (PDB 7ZR5), and AlphaFold models for GC-C (Jumper et al., 2021; Mirdita et al., 2022) were docked into the map using UCSF Chimera X (Pettersen et al., 2021). A resultant hybrid model was then manually curated to contain the correct *Cricetulus griseus* sequences for Hsp90β–Cdc37 and run through Namdinator (Kidmose et al., 2019). This was followed by automated refinement using Phenix real space refine (Adams et al., 2010) and manual building in Coot (Emsley & Cowtan, 2004). The final model produced a favorable MolProbity score of 2.14 (Chen et al., 2010) with 0.4% Ramachandran outliers (Table S1). Model building and refinement software was installed and configured by SBGrid (Morin et al., 2013).

Data Availability

CryoEM maps and atomic coordinates for the GC-C–Hsp90–Cdc37 complex have been deposited in the EMDB (EMD-29523) and PDB (8FX4). Material availability: The plasmids used in this study are available from KCG (kcgarcia@stanford.edu) by request.

Acknowledgements

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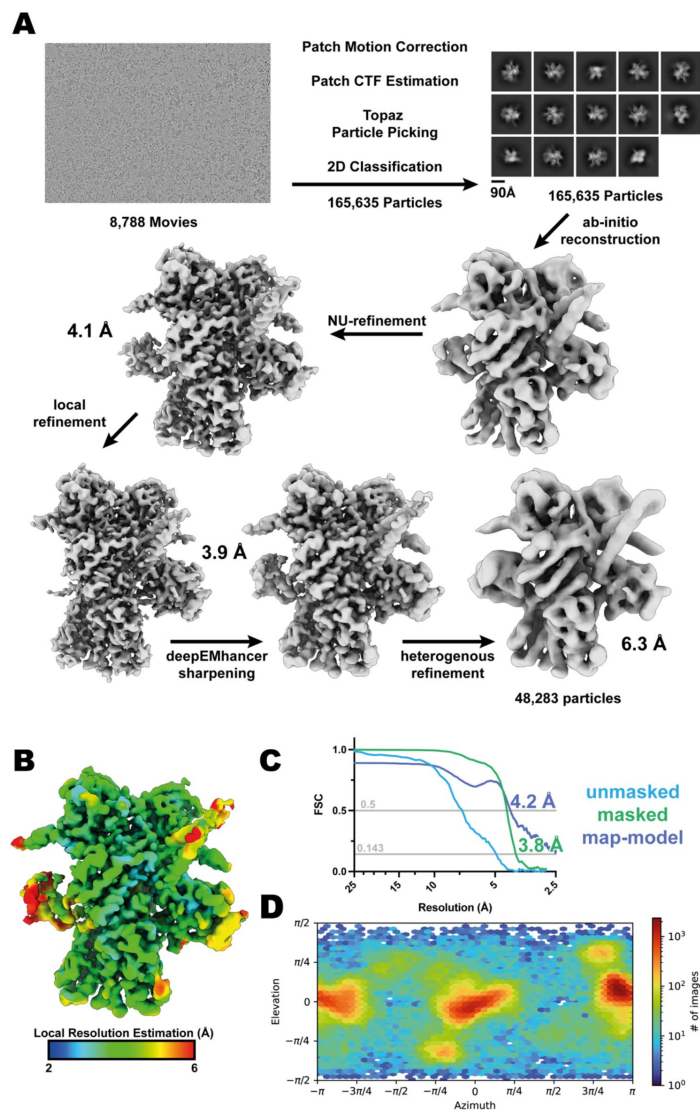
Author Contributions

NAC contributed to conceptualization, methodology, investigation, analysis, writing – original draft, review, and editing. NT contributed to conceptualization, methodology,

investigation, writing – review, and editing. KGC contributed to conceptualization, supervision, writing – review and editing, and funding acquisition.

Competing Interests

The authors declare no competing interests.

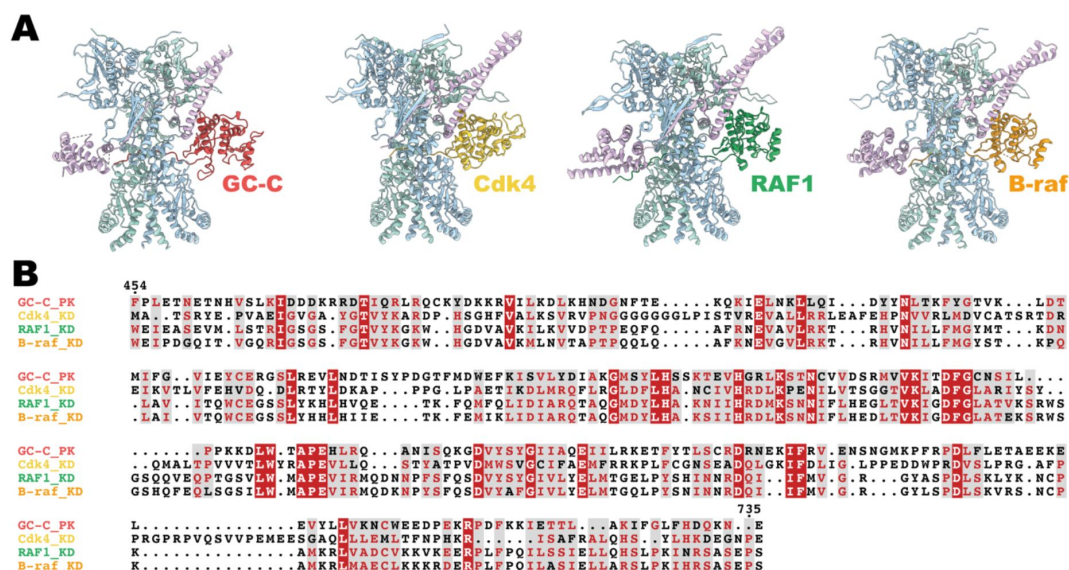


Supplemental Figure 1.

GC-C-Hsp90-Cdc37 complex cryoEM data processing.

(A) Workflow for cryoEM data processing.

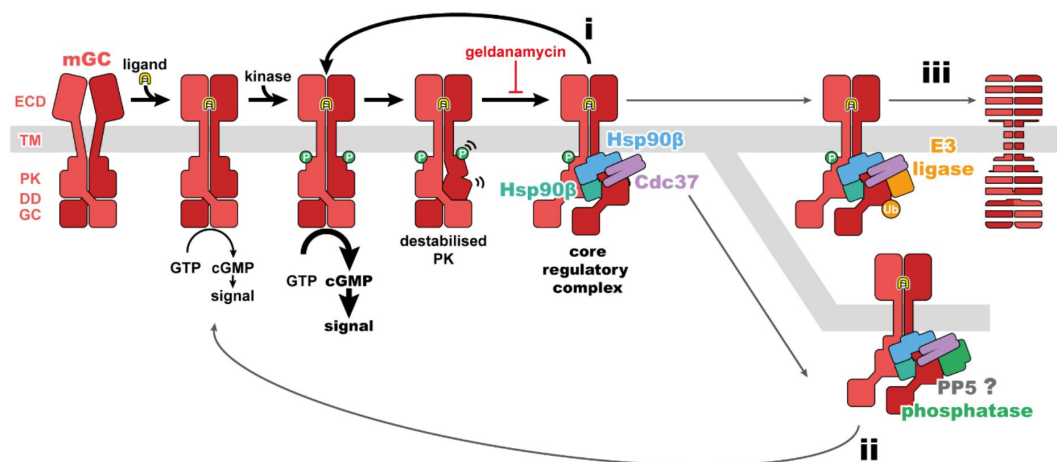
Representative micrograph, reference free 2D averages, and cryoEM maps at the various stages of processing. (B) Local resolution estimation of the finalised cryoEM map. (C) FSC curve of the reconstruction using gold-standard refinement calculated from unmasked and masked half maps. Map-model FSC curve. (D) Orientational distribution of the reconstruction.



Supplemental Figure 2.

Conservation of Cdc37 mediated Hsp90 regulation.

(A) Ribbon representation of a models of client-Hsp90 β -Cdc37 complexes. GC-C is colored in red, Cdk4 in yellow (5FWK), RAF1 in green (7Z37), B-raf in orange (7ZR0), Hsp90 β in light blue and teal, and Cdc37 in light purple. **(B)** A sequence alignment of the pseudokinase domain of GC-C and the kinase domains of Cdk4, RAF1, and B-raf.



Supplemental Figure 3.

Regulatory mechanisms for mGC activity.

A schematic of mGC ligand induced activity, phosphorylation, and destabilization, leading to formation of the mGC– Hsp90–Cdc37 complex structurally characterized in this work. This core regulatory complex would then lead to refolding of the PK and reactivation of the receptor (i), recruitment of PP5 and dephosphorylation of the receptor (ii), or recruitment of E3 ligases and removal of the receptor (iii). An mGC is depicted in red, ligand in yellow, Hsp90 in blue and teal, Cdc37 in purple, a phosphatase in green, and an E3 ligase in orange.

Supplementary Table 1.

	GC-C–Hsp90–Cdc37 complex	
	PDB 8FX4	GC-C–Hsp90–Cdc37 complex
	EMD-29523	with DD density
Data collection and processing		
Magnification		45,000
Voltage (keV)		200
Electron exposure (e/Å ²)		58.8
Defocus range (μm)		0.8 to 2.0
Pixel size (Å)		0.9273
Symmetry imposed		C1
Final particle images	165,635	48,283
Map resolution FSC threshold		0.143
Map resolution (Å)	3.9	6.3
Refinement		
Initial model used (PDB)	5FWK, 7ZR5, AlphaFold	
Model resolution FSC threshold (Å)	0.5	
Model resolution (Å)	4.2	
Model Composition		
Non-hydrogen atoms	13,478	
Protein residues	1,654	
Ligands	2	
B-factors (Å ²)		
Protein	119.49	
Ligand	102.85	
R.m.s. deviations		
Bond lengths (Å)	0.004	
Bond angles (°)	0.914	
Validation		
MolProbity score	2.14	
Clashscore	13.88	
Rotamer outliers (%)	0.67	
Ramachandran plot		
Favoured (%)	92.0	
Allowed (%)	7.6	
Outliers (%)	0.4	

CryoEM data collection, refinement, and validation statistics.

	GC-C–Hsp90–Cdc37 complex	
	PDB 8FX4	GC-C–Hsp90–Cdc37 complex with DD density
	EMD-29523	
Data collection and processing		
Magnification		45,000
Voltage (keV)		200
Electron exposure (e ⁻ /Å ²)		58.8
Defocus range (μm)		0.8 to 2.0
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Reviewer #1 (Public Review):

Membrane receptor guanylyl cyclases are important for many physiological processes but their structures in full-length and their mechanism are poorly understood. Caveney et al.

determined the cryo-EM structure of a highly engineered GC-C in a complex with endogenous HSP90 and CDC37. The structural work is solid and the structural information will be useful for the membrane receptor guanylyl cyclases field and the HSP90 field. However, a detailed characterization of the protein sample is lacking. Moreover, the physiological significance of this structure is not fully exploited by supporting experiments and the mechanistic insight is currently limited.

1. The characterization of the protein sample is lacking. SDS-PAGE would be useful to identify potential proteolysis, leading to the dissociation of GC dimer. Further size-exclusion chromatography would be helpful to estimate the molecular weight of the complex and to determine if only GC-C monomer is purified.
2. The orientation distribution of the particles is not homogenous in Fig. S1D. It would be helpful to present the 3DFSC curve to evaluate the effect of preferred orientation on the reconstruction.
3. Description of protein expression details is lacking. Did the author use transient transfection, stable cell line or virus-mediated transduction?
4. HSP90 binds ATP and is often co-purified with endogenous ATP/ADP. Is there ATP or ADP present in the sample/cryo-EM maps? Is the conformation of NBD similar to ATP-bound HSP90? The author needs to include the description/figures about the nucleotide state of HSP90.
5. The catalytic domains of GC have to be dimerized to perform cyclase function. The presence of only one GC-PK monomer in the cryo-EM structure indicates the structure does not represent an active state of GC. These results suggest the GC expressed in this way is not functional. The authors need to explain why most of the GC protein is trapped in this inactive form.
6. The GC-C construct used here is a highly engineered "artificial" construct, which has not been fully characterized in this work. Does this construct have similar activity as the activated wt GC-C? Does the protein (this engineered construct) expressed in CHO cells show activity?
7. Are the residues on the interface between GC and HSP conserved in other members of membrane receptor guanylyl cyclases? Would mutations on this interface affect the activity of GC?
8. The authors propose that targeting HSP90 would tune the activity of GC. Is there any experimental data supporting this idea?
9. The model in Fig. S3 is largely speculative due to the lack of supporting functional data. In addition, it would be better to change the title to "structure of the protein kinase domain of guanylyl cyclase receptor in complex with HSP90 and cdc37" because the mechanistic insight is limited.

Reviewer #2 (Public Review):

Caveney et al have overexpressed an engineered construct of the human membrane receptor guanyl cyclase GC-C in hamster cells and co-purified it with the endogenous HSP90 and CDC37. They have then determined the structure of the resultant complex by single particle cryoEM reconstruction at sufficient resolution to dock existing structures of HSP90 and CDC37, plus an AlphaFold model of the pseudo-kinase domain of the guanylyl cyclase. The novelty of the work stems from the observation that the pseudo-kinase domain of GC-C associates with CDC37 and HSP90 similarly to how the bona fide protein kinases CDK4, CRAF and BRAF have been previously shown to interact.

The experimentation is limited to the cryoEM analysis, and is lacking additional studies that would give deeper insight into the oligomeric nature - if any - of the GC-C when bound to HSP90-CDC37 as compared to the free protein. This is relevant, as the dimerization domain downstream of the pseudokinase, is evident in the maps - albeit not well resolved - and it is not clear whether it is still able to mediate dimerization with a second free or HSP90-CDC37-bound GC-C. It would also be good to see some experimentation that asks whether association with HSP90-CDC37 inhibits the guanyl cyclase activity. It is clear from previous work that HSP90-CDC37 silence the kinase activity of their bound client kinases, but in this case the catalytic guanyl cyclase is not directly associated with the chaperone complex and may still be able to function.

Although the sequence alignment presented in SuppFig 2 shows that GC-C conserves the classic DFG motif that plays a critical role in the regulation of most kinases, the numbering of the sequence is absent, making it very difficult to relate this to the structural detail shown in Fig 2B. This needs to be clarified, as the interaction of CDC37-Trp31 with the DFG motifs and downstream activation loops in CRAF and BRAF have been proposed as important features of the selectivity of these kinases for the HSP90-CDC37 system, and it would be good to be able to see clearly how much of this is also conserved in the GC-C pseudokinase domain interaction. For example, is the much shorter activation segment (DFG -> APE) ordered in the complex or disordered?

It was not easy to follow what was in the sample used for cryoEM. The cloning of the guanylyl cyclase (GC) component is described in the methods and they have shown some illustrations in fig 1 but a proper numbered figure of the domain organisation clearly showing domain boundaries and linker segments is really needed for a reader not familiar with the structure of GCs, especially since they have replaced the ECD with a leucine zipper in their construct. It is important to show a domain figure of what this construct looks like as well, as from the illustrations in fig 1 for examples its hard to see what's PK, DD, GC domains. It would also be helpful to see in the supplementary a gel of complex they put on the grids, to make it clearer what exactly the sample is and to reassure that the GC-C domains that are not resolved in the cryoEM are nonetheless present in the sample.

Overall there is only minimal proposal of mechanism or biological function based on the structure. The speculation in the Discussion of two fates - PP5 dephosphorylation or E3 ligase recruitment, is not supported by any experimentation, which is reasonable for speculation, but is also not underpinned by reference to any previously published work suggesting that these additional processes may be important. In the absence of any work by the authors can they put these speculations more in context with previously published work that supports the importance of these processes specifically for GC regulation?

Reviewer #3 (Public Review):

A detailed understanding of how membrane receptor guanylyl cyclases (mGC) are regulated has been hampered by the absence of structural information on the cytoplasmic regions of these signaling proteins. The study by Caveney et al. reports the 3.9Å cryo-EM structure of the human mGC cyclase, GC-C, bound to the Hsp90-Cdc37 chaperone complex. This structure represents a first view of the intracellular functional domains of any mGC and answers without doubt that Hsp90-Cdc37 recognizes mGCs via their pseudokinase (PK) domain. This is the primary breakthrough of this study. Additionally, the new structural data reveals that the manner in which Hsp90-Cdc37 recognizes the GC-C PK domain C-lobe is akin to how kinase domains of soluble kinases docks to the chaperone complex. This is the second major finding of this study, which provides a concrete framework to understand, more broadly, how Hsp90-Cdc37 recruits a large number of other diverse client proteins containing kinase or pseudokinase domains. Finally, the Hsp90-Cdc37-GC-C structure offer clues as to how GC-C

may be regulated by phosphorylation and/or ubiquitinylation by serving as a platform for recruitment of PP5 and/or E3 ligases.

Comments:

1. The authors used an interesting approach to obtain the GC-C-Hsp90-Cdc37 complex. Flag-tagged human GC-C was overexpressed in CHO cells with the expectation of co-purifying endogenous hamster homologs of Hsp90 and Cdc37. There are several points worth noting:
 - a. It is not clear from the data presented (Figure 1C, Suppl Fig 1A) or the Methods the percentage of particles in the cryo-EM specimen that represent the GC-C-Hsp90-Cdc37 complex. Presumably, some fraction of GC-C isolated will not be associated with Hsp90-Cdc37. If a very large portion of GC-C is associated with Hsp90-Cdc37, it would be good to explain why this is to be expected. Are 2D/3D classes corresponding to the activated GC-C dimer found? If not, why?
 - b. Figure 1A suggests that GC-C is phosphorylated before recruitment of Hsp90-Cdc37. What is the phosphorylation status of the GC-C specimen that was imaged by cryo-EM?
 - c. The resolution of the cryo-EM map (3.9 Å) is too low for unambiguous identification of proteins. Please provide more precise justification for the claim that the densities observed do in fact correspond to hamster Hsp90 and Cdc37.
 - d. The authors state that human GC-C pulls down hamster Hsp90-cdc37 but soluble kinases cannot, despite the high sequence identity between human and hamster Hsp90-cdc37. Is this because GC-C recognition is more promiscuous? Can this difference be understood in light of the new structural information presented?
2. A large portion of the enforced GC-C dimer was not visible in the cryo-EM maps. It is not easy to learn from Figure 1 exactly which parts of the GC-C construct was sufficiently ordered and observed structurally. Please improve Figure 1.
3. On page 4, the authors claim that they are able to orient the GC-C-Hsp90-Cdc37 complex "as it would sit on a membrane" and referred to Figure 1B. It is not clear what is implied here. Does Hsp90-Cdc37 binding constrain the complex to face the inner leaflet of the membrane in a specific orientation as shown in Figure 1B? If true, this could potentially have important functional implications. Please illustrate how this was deduced based on the information available.
4. Also on page 4, it is stated that it is sterically unlikely an additional Hsp90-Cdc37 complex is associated with the other copy of GC-C in the leucine zippered dimer. It is not obvious to the reader how this may be the case. An additional figure could help make this more clear. Additional biochemical evidence will also help. The absence of GC-C-Hsp90-Cdc37 dimers in cryo-EM micrographs can also support the argument.
5. Some comments on Figure 2:
 - a. NTD and CTD are mislabeled in Figure 2A.
 - b. The authors should show cryo-EM density to support their modeling of GC-C in Figures 2B and C.
6. The authors claim that Hsp90-Cdc37 clients are more similar structurally near the cdc37 interface. Please illustrate this with additional figures. Suppl. Figure 2 is inadequate for this purpose. The authors can also consider adding a more detailed discussion comparing the interactions between the pseudokinase/kinase C-lobe and Cdc37 in known structures. Is shape/charge complementarity a universal feature of cdc37-dependent kinase/pseudokinase recruitment? It would be interesting to also consider if it would be possible to predict which of the ~60 human pseudokinases are possible Hsp90-Cdc37 clients. New structural findings from this study and publicly available AI-predicted protein structures could help.