Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide

Arun K. Shukla1*, Aashish Manglik2*, Andrew C. Kruse2*, Kunhong Xiao1, Rosana I. Reis1, Wei-Chou Tseng1, Dean P. Staus1, Daniel Hilger2, Serdar Uysal3, Li-Yin Huang1, Marcin Paduch3, Prachi Tripathi-Shukla1, Akiko Koide3, Shohei Koide3, William I. Weiss4,5, Anthony A. Kossiakoff6, Brian K. Kobilka7& Robert J. Lefkowitz5,6

The functions of G-protein-coupled receptors (GPCRs) are primarily mediated and modulated by three families of proteins: the heterotrimeric G proteins, the G-protein-coupled receptor kinases (GRKs) and the arrestins1. G proteins mediate activation of second-messenger-generating enzymes and other effectors, GRKs phosphorylate activated receptors2, and arrestins subsequently bind phosphorylated receptors and cause receptor desensitization1. Arrestins activated by interaction with phosphorylated receptors can also mediate G-protein-independent signalling by serving as adaptors to link receptors to numerous signalling pathways1. Despite their central role in regulation and signalling of GPCRs, a structural understanding of β-arrestin activation and interaction with GPCRs is still lacking. Here we report the crystal structure of β-arrestin-1 (also called arrestin-2) in complex with a fully phosphorylated 29-amino-acid carboxy-terminal peptide derived from the human V2 vasopressin receptor (V2Rpp). This peptide has previously been shown to functionally and conformationally activate β-arrestin-1 (ref. 5). To capture this active conformation, we used a conformationally selective synthetic antibody fragment (Fab30) that recognizes the phosphopeptide-activated state of β-arrestin-1. The structure of the β-arrestin-1–V2Rpp–Fab30 complex shows marked conformational differences in β-arrestin-1 compared to its inactive conformation. These include rotation of the amino- and carboxy-terminal domains relative to each other, and a major reorientation of the ‘lariat loop’ implicated in maintaining the inactive state of β-arrestin-1. These results reveal, at high resolution, a receptor-interacting interface on β-arrestin, and they indicate a potentially general molecular mechanism for activation of these multifunctional signalling and regulatory proteins.

Binding of β-arrestins to phosphorylated GPCRs is thought to involve two types of interaction between a receptor and a β-arrestin molecule6. A phosphate sensor engages the phosphorylated carboxy terminus or third intracellular loop of the receptor, and a conformational sensor recognizes the agonist-induced, active conformation of the core of the receptor (Fig. 1a). Using mass-spectrometry-based conformational mapping, we have previously used a V2 vasopressin-receptor-derived phosphopeptide (V2Rpp) to investigate activation of β-arrestin-1 and β-arrestin-2 (also known as arrestin-3)5,7. Binding to V2Rpp recapitulates functionalities of receptor-activated β-arrestins, such as enhanced clathrin binding7. Thus, we reasoned that crystallographic study of a complex of β-arrestin-1 with V2Rpp would provide insight into the mechanisms of receptor-mediated β-arrestin activation. However, well-ordered crystals of β-arrestin-1 bound to V2Rpp could not be obtained. This is presumably due to the significant conformational flexibility of activated arrestin molecules, as was recently determined for visual arrestin (also called arrestin-1) by NMR spectroscopy8. Given the success of antigen binding fragments (Fabs)9 and nanobodies10 in stabilizing particular GPCR conformations, we sought to identify and characterize conformationally selective Fabs that stabilize the V2Rpp bound, active conformation of β-arrestin-1.

We used a minimalist synthetic Fab phage display library11 to select several high-affinity Fabs that selectively recognize the β-arrestin-1–V2Rpp complex (Supplementary Fig. 1). One of these, Fab30, displays marked selectivity for the activated conformation of β-arrestin-1 induced by V2Rpp (Fig. 1b). To ensure that Fab30 stabilizes a physiologically relevant conformation of β-arrestin-1, we investigated whether this Fab could facilitate interaction between a receptor and β-arrestin-1. Here, we used the previously described chimae receptor β2-V2R which has an identical C terminus to V2Rpp, and which also has unaltered ligand-binding characteristics compared to the wild-type β2 adrenergic receptor (β2AR)12. Complexes of GPCRs with either G proteins or β-arrestins display an enhanced affinity for agonists due to the allosteric interactions among the agonist, the receptor and the transducer (G protein or β-arrestin)13,14. Addition of exogenous β-arrestin-1 to the membranes containing phosphorylated β2-V2R resulted in a small fraction of the receptor in a high-agonist affinity state compared to receptor alone (Fig. 1c). Addition of Fab30 significantly increased the percentage of receptors in the high-affinity state. Furthermore, a direct physical stabilization of the receptor–β-arrestin-1 complex by Fab30 was revealed by co-immunoprecipitation (Fig. 1d). Here we present a 2.6 Å crystal structure of the β-arrestin-1–V2Rpp–Fab30 complex (Fig. 1e).

The overall structure of activated β-arrestin-1 exhibits a wide variety of pronounced structural changes compared to previously determined inactive state structures. Most notably, the N and C domains of β-arrestin-1 undergo a substantial twist relative to one another (Fig. 2a, b), with a 20° rotation around a central axis. The V2Rpp binds to the N domain at a similar location to the β-arrestin-1 C terminus in inactive structures and makes extensive contacts, primarily through charge–charge interactions of V2Rpp phosphates with β-arrestin-1 arginine and lysine side chains (compare Fig. 2c with Figs 2d and 3d).

This binding mode is consistent with previous limited proteolysis studies that revealed protection of the N domain of β-arrestin-1 in the presence of V2Rpp. Additionally, crosslinking experiments on the β-arrestin-1–V2Rpp complex in the absence of Fab30 show that the N terminus of V2Rpp is in close proximity to K77, consistent with our structure (Supplementary Fig. 2). Like the β-arrestin-1 C terminus, V2Rpp binds β-arrestin-1 by extending the N-domain β-sandwich fold. Unlike the C terminus, however, V2Rpp binds as an antiparallel β-strand. This binding mode may serve as a general mechanism by which arrestins recognize the phosphorylated loops and C-terminal tails of receptors.

In addition to the large interdomain rearrangement, the N domain and central loops show large structural changes associated with

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β-arrestin-1 activation. Several loops have been implicated in various aspects of β-arrestin activation and receptor interaction. These include the 'finger loop' (residues 63–75), the 'middle loop' (residues 129–140) and the 'lariat loop' (residues 274–300). Each of these loops exhibits activation-dependent conformational changes (Fig. 2c–e). Comparison of these loops with inactive structures of β-arrestin-1 shows the considerable flexibility in each loop in the inactive conformation, but a more marked change in conformation upon activation (Fig. 2e). The crystal structure reveals that the V2Rpp occludes the inactive conformation of the finger loop, which has been shown to be important for arrestin discrimination between active and inactive states. Disruption of this interaction by mutagenesis yields arrestins that are partially phosphorylation-independent in their binding to receptor, termed the 'high agonist affinity state'. Therefore, the fraction of receptor in the high-agonist affinity state reflects the extent of complex formation between receptor and β-arrestin. In a radioligand competition binding assay using [125I]CYP as the probe and the agonist isoproterenol (Iso) as the competitor, β-arrestin-1 alone shifts a small portion (14%) of receptors into the high agonist affinity state. Fab30 significantly amplifies this effect (31%) (n = 3, P < 0.0001 in F test). In a pull-down assay, phosphorylated β-V2R chimaera shows appreciable binding to β-arrestin-1 only in the presence of Fab30. Overall structure of the β-arrestin-1–V2Rpp–Fab30 complex.

**Figure 1** | Fab30 specifically recognizes and stabilizes an active state of β-arrestin-1. a, GPCRs are phosphorylated after activation, leading to the binding of arrestins. Interactions between the phosphorylated receptor and β-arrestin-1 lead to β-arrestin-1 activation and the subsequent blockade of G-protein signalling and initiation of β-arrestin-1 signalling pathways. b, Interaction between β-arrestin-1 and Fab30 requires the presence of V2Rpp in a size exclusion assay. c, The formation of a complex between a GPCR and β-arrestin allosterically leads to an enhanced affinity of agonist for the receptor, termed the 'high agonist affinity state'. Therefore, the fraction of receptor in the high-agonist affinity state reflects the extent of complex formation between receptor and β-arrestin. In a radioligand competition binding assay using [125I]CYP as the probe and the agonist isoproterenol (Iso) as the competitor, β-arrestin-1 alone shifts a small portion (14%) of receptors into the high agonist affinity state. Fab30 significantly amplifies this effect (31%) (n = 3, P < 0.0001 in F test). d, In a pull-down assay, phosphorylated β-V2R chimaera shows appreciable binding to β-arrestin-1 only in the presence of Fab30. e, Overall structure of the β-arrestin-1–V2Rpp–Fab30 complex.
V2Rpp and displacement of the C terminus does not markedly alter the secondary structure of β-strand I. Although we observe abundant charge-charge interactions between β-arrestin-1 and V2Rpp, it is noteworthy that neither the specific sequence of the phosphorylation sites nor the net number of phosphates is conserved among various receptors. Therefore, it remains to be seen how β-arrestins fine-tune their interaction with such a large number of receptors.

The second constraint that stabilizes the inactive conformation of arrestins is the polar core\(^1\), consisting of five interacting charged residues: D26, R169, D290, D297 and R393. Disruption of the polar core by mutagenesis yields phosphorylation-independent mutants of both visual arrestin and β-arrestin-1 (refs 17, 21). Charge reversal of R169 or D290 in β-arrestin-1 (R175 and D296 in visual arrestin) disrupts this interaction network, yielding arrestins that can bind non-phosphorylated, activated receptors. On the basis of these studies, R169 was previously proposed to be a critical phosphate sensor in β-arrestin-1, and disruption of the polar core was proposed to be required for β-arrestin-1 activation\(^2\). Contrary to this model, R169 does not make any direct contacts with V2Rpp phosphates, indicating that direct interaction between R169 and receptor phosphates is not required for arrestin activation (Fig. 3e, f). However, binding of V2Rpp does disrupt the polar core. V2Rpp binding to β-arrestin-1 displaces the arrestin C terminus, and in doing so, removes R393 from the polar core. Residues D290 and D297 also lose interactions within the polar core, and this is accompanied by a marked twisting of the lariat loop, which contains both D290 and D297. Therefore, it is possible that the disruption of the polar core is driven by the excess negative charge in this region following displacement of the arrestin C terminus residue R393. Notably, the side chain of K294, a residue within the lariat loop, flips towards the N domain upon activation and engages pT360. It is possible that K294 recognition of phosphates provides an additional driving force for lariat-loop rearrangement, and may therefore stabilize β-arrestin-1 in an active conformation. This observation in the crystal structure is consistent with crosslinking experiments, which

Figure 2 | Conformational changes associated with β-arrestin-1 activation. The structures of inactive β-arrestin-1 (Protein Data Bank (PDB) accession 1G4M; chain A, light blue) and active β-arrestin-1 (gold) were aligned on the N domains. The β-arrestin-1 C terminus is highlighted in dark blue. a, A substantial rotation and translation of the C domain relative to the N domain occurs upon activation. The rotation axis is indicated as a solid black line. b, View of C-domain rotation along the axis. c, N domain of inactive arrestin, highlighting important regions. d, Active β-arrestin-1 in the same orientation, showing V2Rpp in green. Phosphorylated residues are highlighted as sticks. e, The overall structure of inactive β-arrestin-1 (PDB 1G4M; chain A), with loops from all inactive β-arrestin-1 structures superimposed (grey loops). The active conformation of these loops (orange loops) deviates from all inactive structures.
reveal the disappearance of an intrapeptide crosslink between K292 and K294 in the presence of V2Rpp (Supplementary Fig. 5), indicating that V2Rpp induces a conformation like that seen in the crystal structure even in the absence of Fab30.

Whereas domain rearrangement upon arrestin activation has been proposed previously, the observed 20° twisting of the N and C domains of β-arrestin-1 upon activation is unanticipated. Biochemical studies have shown that sequential deletion of the visual arrestin hinge region connecting the N and C domains results in a progressive decrease in the ability of arrestin to bind phosphorylated, light-activated rhodopsin. This suggests a requirement for relative movement of the two domains for efficient interaction with activated receptors. Considering the large number of interaction partners of β-arrestins during cellular signalling, it is tempting to speculate that the twisting movement of the two domains upon arrestin activation may expose interaction interfaces with such binding partners.

Figure 3 | V2Rpp interactions with β-arrestin-1. a, Overall view of β-arrestin-1, with regions of interest in boxes. b, V2Rpp (green) displaces the inactive finger loop (light blue), causing it to adopt an extended conformation in the active state (gold). Select charge–charge contacts are shown with dotted lines in b–f. c, In the inactive conformation, the β-arrestin-1 C-terminal β-strand (dark blue) lies along the N domain in the three-element interaction network. d, Upon activation, this strand is displaced by the C terminus of V2Rpp, which engages in extensive charge–charge interactions through phosphorylated residues. e, The polar core of β-arrestin-1 is thought to be a critical stabilizer of the inactive state. f, Upon V2Rpp binding, the C-terminal strand residue Arg 393 is displaced, and its interaction partner D297 undergoes a large movement together with the rest of the lariat loop.
phosphorylated rhodopsin is also evident in the crystal structure of activated β-arrestin-1. Given the importance of this region in maintaining the inactive conformation of visual arrestin, the agreement in conformational changes within arrestin suggests that the V2Rpp-bound, active conformation of β-arrestin-1 presented here represents a similar state to that of arrestin in complex with a phosphorylated, activated GPCR. This further suggests that the conformational changes associated with activation and receptor binding are conserved throughout the arrestin family. However, the binding stoichiometry between GPCRs and arrestins still remains to be fully established. Recent biochemical studies have suggested that two rhodopsin molecules may simultaneously bind one arrestin. This extensive and specific contacts between V2Rpp and the β-arrestin-1 N domain probably preclude another receptor C terminus from binding β-arrestin-1. However, it is possible that an arrestin molecule bound to the phosphorylated C terminus of a receptor could interact with the seven-transmembrane-segment core of another receptor. Additional data, including a crystal structure of a GPCR–β-arrestin complex, will be required to be clarified.

In summary, we present here the structure of an activated arrestin bound to the phosphorylated C terminus of a GPCR. The structure not only provides the atomic details of a potentially general GPCR–β-arrestin interaction interface, but also offers novel insights into the activation process of arrestins, and reveals a large interdomain twisting associated with activation. These findings will facilitate future efforts to understand the structural basis for β-arrestin activation and signalling. Such studies may ultimately yield insight into how GPCRs achieve such a large breadth of signalling complexity.

METHODS SUMMARY

A truncated version of β-arrestin-1 containing residues 1–393 was expressed in Escherichia coli strain BL21(DE3) and purified to homogeneity using a GST tag and anion exchange chromatography. Fab30 was expressed in E. coli strain S254 and purified by protein A and cation exchange chromatography. Mixture and incubation of the components yielded the β-arrestin–1–V2Rpp–Fab30 complex, which was purified by size-exclusion chromatography and crystallized using vapour diffusion. Diffraction data were collected at GM/CA-CAT beamline which was purified by size-exclusion chromatography and crystallized using vapour diffusion. Diffraction data were collected at GM/CA-CAT beamline 23ID-D at the Advanced Photon Source at Argonne National Laboratory.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions A.K.S. conceived the project, designed the Fab selection strategy, selected and characterized Fab30, established and optimized complex formation and purification conditions, prepared protein for crystallization trials and supervised the experiments related to the biochemical characterization of the complex. A.M. purified the complex, performed crystallography trials and grew crystals. A.M. and A.C.K. collected and processed diffraction data, and solved and refined the structure with supervision from W.I.W. R.I.R. assisted with advanced Fab characterization and optimized complex formation. W.-C.T. assisted with Fab selection and preliminary characterization. K.K. performed and analysed the crosslinking experiments. D.P.S. performed and analysed radioligand binding experiments. L.-Y.H. assisted with functional characterization of the complex. P.T.-S. expressed and purified the receptor. S.U., M.P., A.K., S.K. and A.A.K. generated and provided the phage display library and the screening protocol and helped with the initial phase of Fab selection. D.H. performed the comparison of the structural model with EPR data. A.K.S., A.M. and A.C.K. made figures. A.K.S., AM, A.C.K., B.K.K. and R.J.L. wrote the manuscript. B.K.K. and R.J.L. supervised the overall research.

Author Information Coordinates and structure factors for the β-arrestin–1–V2Rpp–Fab30 complex are deposited in the Protein Data Bank under accession code 4JQI. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this paper. Correspondence and requests for materials should be addressed to B.K.K. (bokilla@stanford.edu) or R.J.L. (lefko001@receptor-biol.duke.edu).
METHODS

Purification of β-arrestin-1. Full-length β-arrestin-1 was purified from E. coli as described previously27. Briefly, GST-tagged rat β-arrestin-1 in the pGEX4T vector was transformed into BL21(DE3) cells, large-scale expression cultures were grown in Terrific broth, and induced with 1 m IPTG for 16 h at 16 °C. Cell pellets were lysed in 20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM PMSF and 2 mM dithiothreitol (DTT) using a micro fluidizer, and the lysate was bound to glutathione sepharose at 4 °C for 2 h. Beads were washed in lysis buffer and β-arrestin-1 was eluted by overnight incubation with thrombin at 4 °C. β-arrestin-1 was then purified with a HiTrap Q column and eluted by a linear gradient of NaCl. Peak fractions were pooled and purified protein was dialysed in 20 mM HEPES pH 7.4 and 150 mM NaCl.

Selection and characterization of Fab. The phage library was panned against biotinylated β-arrestin-1 bound to V2Rpp and immobilized on streptavidin beads. Beads were washed three times and bound phages were amplified by infecting E. coli XL-1 blue cells. Amplified phage were precipitated and used for a second and third round of panning. To select against Fab that bind to the inactive conformation of β-arrestin-1, beads coated with the β-arrestin-1–V2Rpp complex were first incubated with phage and then with 1 mM non-biotinylated β-arrestin-1. Subsequently, phage were eluted with dithiothreitol (DTT) and resulting clones were used for single point ELISA to test their selectivity towards β-arrestin-1 bound to V2Rpp. ELISA positive clones were sequenced and further characterized.

Radioligand binding. S9 insect cells were co-infected with baculovirus encoding an N-terminal Flag-tagged βv2-V2R (a chimaeric receptor with βAR residues 1–341 and V2 vasopressin receptor residues 328–372) and GRK2-CAAAX (GRK2 with a membrane tethering prenylation signal). After viral infection for 72 h at 27 °C, cells were incubated with 10 μM isoproterenol at 37 °C for 15 min to induce receptor phosphorylation. Subsequently, the cells were washed and membranes were prepared and flash frozen. Membranes were extensively washed to remove isoproterenol used for receptor phosphorylation. For radioligand binding, membranes were incubated with 60 μM [125I]cyanopindolol (GE Healthcare Life-Science) in radioligand binding buffer (50 mM Tris, pH 7.4, 50 mM potassium acetate, 0.5 mM magnesium chloride, 1 mM ascorbic acid) with varying concentrations of freshly prepared isoproterenol. Binding reactions were performed in parallel, with 1 μM β-arrestin-1 (residues 1–393) incubated either in the presence or absence of 10 μM Fab30. Binding reactions were incubated for 90 min at 25 °C, followed by rapid harvesting on a GF-B filter and scintillation counting in a Beckman scintillation counter. Competition binding data were analysed by a nonlinear curve-fitting procedure where low- and high-affinity values were computed globally using a two-site binding model (GraphPad Prism). The F-test was used to test whether Fab30 significantly altered the amount of βv2-V2R coupled to β-arrestin.

Effect of Fab30 on βv2-V2R–β-arrestin-1 interaction. Fab30 was expressed and purified as described previously27. βv2-V2R was expressed in S9 cells and purified as described previously27. Purified, phosphorylated βv2-V2R was prepared bound to the potent βAR agonist BI-16710228 and incubated at a concentration of 1 μM with 3 μM β-arrestin-1 with and without Fab30 at 25 °C for 2 h in a buffer comprised of 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.01% MNG (lauryl maltose neopentyl glycol). Subsequently, βv2-V2R was immunoprecipitated using M1 Flag antibody beads. Beads were washed and protein was eluted with 5 mM EDTA and 0.25 mg/mL Flag peptide and elution fractions were analysed on a 4–20% SDS–PAGE gel and stained with Coomassie.