Label-free quantification of calcium-sensor targeting to photoreceptor guanylate cyclase and rhodopsin kinase by backscattering interferometry

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Quantification of protein binding to membrane proteins is challenging and a limited set of methods is available to study such systems. Here we employed backscattering interferometry (BSI), a free-solution label-free method with high sensitivity, to quantify the interaction of neuronal Ca2+ Sensor proteins with their targets operating in phototransduction. We tested direct binding of guanylate cyclase–activating proteins (GCAP1 and GCAP2) to their membrane target guanylate cyclase 1. The regulatory mechanism of GCAPs including their binding interface in the target is unresolved. Here we used a label-free, free-solution assay method based on BSI to determine binding constants of GCAP1 and GCAP2 to the full-length membrane-bound guanylate cyclase type 1. GCAP1 and GCAP2 bound to different regions on the target guanylate cyclase with submicromolar affinity (apparent K\textsubscript{D}-values of $663 \pm 121 \text{ nM}$ and $231 \pm 63 \text{ nM}$ for Ca\textsuperscript{2+}-free GCAP1 and GCAP2, respectively). A guanylate cyclase construct containing the juxta-membrane and kinase homology domain harbored an exclusive binding site for GCAP1 with similar affinities as the full-length protein, whereas GCAP2 did not bind to this region. We provide a model in which GCAP1 and GCAP2 do not share a single binding site to the target, thus cannot exchange upon fluctuating Ca\textsuperscript{2+} levels.

Photoreceptor cells efficiently respond to changing light conditions on a millisecond time scale by a well-balanced interplay of two second-messenger species, cGMP and calcium1–3. Light excitation of the G protein-coupled receptor rhodopsin triggers a downstream signaling cascade leading to hydrolysis of cGMP, subsequently leading to a decrease of cytoplasmic Ca\textsuperscript{2+}-concentration [Ca\textsuperscript{2+}]. A network of Ca\textsuperscript{2+}-sensor proteins in rod and cone photoreceptor cells can detect subtle changes in intracellular [Ca\textsuperscript{2+}]. These Ca\textsuperscript{2+}-sensors precisely regulate the enzymatic activity of their targets providing an efficient Ca\textsuperscript{2+}-mediated feedback loop to restore second messenger levels prior to illumination. Among them the guanylate cyclase-activating proteins (e.g. GCAP1 and GCAP2 in mammalians) control the synthesis of GMP by sensory guanylate cyclases (GCs) in a Ca\textsuperscript{2+}-dependent manner and in a step-by-step Ca\textsuperscript{2+}-relay mechanism. GCAPs contain three functional and one non-functional EF-Hand type of Ca\textsuperscript{2+}-binding motifs and are Ca\textsuperscript{2+}-sensor proteins belonging to the family of neuronal Ca\textsuperscript{2+}-sensor (NCS) proteins4–7. Mammalian express two or three, while teleost fish express six to eight different GCAP isoforms in their rod and cone cells, which all regulate enzymatic activity of sensory GCs at different Ca\textsuperscript{2+} levels. Synthesis of cGMP under control of cytoplasmic Ca\textsuperscript{2+} contributes to the restoration of the dark adapted state and mediates light adaptation of photoreceptors1–3.

Mammalian rod and cone cells express two forms of a sensory GC, assigned as GC-E and GC-F (alternatively named ROS-GC1 or 2, retGC1 or 2). Both GCs form homo-dimers in disc membranes of rod and cone outer segments, but recent research was mainly focused on GC-E, probably for the following reasons: (i) the fraction of total GC-E exceeds that of GC-F by 25-fold in bovine10 and 4-fold in mouse outer segments11; (ii) various
Retinal diseases like Leber’s congenital amaurosis (LCA) and Cone-Rod-Dystrophies (CORD) correlate with mutations in the \textit{GUCY2D} gene coding for photoreceptor GC-E12; (iii) mutations in the \textit{GUCA1A} gene coding for GCAP1 correlate with cone, cone-rod and macular dystrophies in patients suffering from visual dysfunction, which is likely caused by an impaired operation of the GC-E/GCAP1 complex, since GC-E is the preferred target of GCAP113.

Deciphering the control mechanisms of cGMP synthesis in the GC-E/GCAP1 complex is important in understanding cGMP homeostasis in photoreceptor cells in health and disease. Previous work aimed at identifying the interaction sites of GCAP1 and GCAP2 in GC-E has led to inconsistent, even contradictory results 14–19. While several studies indicated distinct binding sites for GCAP1 and GCAP2 localized in different regions in the cytoplasmic part of GC-E14–16,19, more recent studies suggested a differential activation mechanism in which GCAPs exchange at a shared single binding site upon fluctuating Ca\(^{2+}\) concentration17,18.

In the present study we investigated GC-E/GCAP interactions using a novel technique called Backscattering Interferometry (BSI), which allows label- and immobilization-free interaction analysis in physiologically relevant matrices at high sensitivity and in small volumes20–22. Using full-length human GC-E derived from heterologous expression in HEK 293 cells we quantified the binding of GCAP1 and GCAP2 to GC-E by BSI. It was also possible to test both of the currently proposed binding models: one, in which GCAP1 and GCAP2 interact at different sites in GC-E, and the other one where both NCS proteins share a single binding site. Control BSI assays were performed with the photoreceptor NCS protein recoverin that is known to bind to rhodopsin kinase GRK1 in a Ca\(^{2+}\) dependent manner23–27, enabling the determination of specific affinity in this complex physiological system.

Results and Discussion

Binding of Recoverin to GRK1. BSI assays are relatively new and must be performed as relative measurements23. Therefore, we first employed recoverin as a test system for BSI performance benchmarking. Recoverin was chosen because, like GCAP1 and 2, it belongs to the family of NCS proteins and it exhibits similar three-dimensional folding5,7. It interacts with the N-terminal 25 amino acids of rhodopsin kinase (GRK1) thereby controlling its activity in a Ca\(^{2+}\)-dependent manner. Here we quantified the binding affinity of recoverin to the N-terminal GRK1 fragment (NRK) employing the same GST fusion protein used earlier24. When increasing concentrations of recoverin were mixed with NRK at saturating Ca\(^{2+}\), BSI reported a \(K_D\) of 4.72 \(\pm\) 0.72 \(\mu\)M (number of replicates \(n = 4\)). In the absence of Ca\(^{2+}\) (EGTA) no binding signal was observed (\(n = 5\)). BSI data was fitted to a simple one site ligand binding model to obtain the \(K_D\) value.

![Figure 1. Interaction of recoverin (Rec) with the N-terminal GRK1 fragment (NRK) by BSI. A dilution series with increasing concentration of myristoylated bovine recoverin was prepared with a constant concentration of a GST fusion protein (4 \(\mu\)M) containing the N-terminal 25 amino acids of bovine GRK1. In the presence of 200 \(\mu\)M Ca\(^{2+}\) BSI reported a \(K_D\) of 4.72 \(\pm\) 0.72 \(\mu\)M (number of replicates \(n = 4\)). In the absence of Ca\(^{2+}\) (EGTA) no binding signal was observed (\(n = 5\)). BSI data was fitted to a simple one site ligand binding model to obtain the \(K_D\) value.](image-url)
The binding of GCAPs to its target is Ca\(^{2+}\) independent. Although numerous studies covered the topic of localizing GCAP binding sites in photoreceptor GC-E in the last 20 years, the results are inconclusive\(^{15-19}\) in terms of defining the GCAP1/GC-E interaction interface. It is unclear as to why these experiments diverge, but the use of different experimental approaches might have led to contradicting results. Here, for the first time, we used a completely label-free experimental set-up to directly study the interaction of purified GCAP1 and GCAP2 with human GC-E.

HEK cell membrane vesicles containing full-length active human GC-E with an average vesicle size distribution of around 50 nm were titrated with increasing GCAP concentrations. Phase changes from BSI recordings which signal chemical interactions were performed in the absence of Ca\(^{2+}\) and saturated between 5–10 μM of GCAP1 or GCAP2 (Fig. 2a,b) resulting in halfmaximal apparent $K_D$-values of 663 ± 121 nM and 231 ± 63 nM, respectively. Both GCAP forms bind in the submicromolar range to the target GC in agreement with previously performed enzyme activation assays using a native GC-E source\(^{28-30}\). However, due to the inhibition of mammalian GC-E by GCAPs in the presence of Ca\(^{2+}\), apparent affinity constants (EC\(_{50}\)) from enzyme activation assays are difficult to obtain and prone to large experimental errors. Yet, this observation is not a limitation for a BSI assay.

When performing the BSI titration in the presence of Ca\(^{2+}\) (Fig. 2c,d) we quantified affinity constants for GCAP1 ($K_D = 505$ nM ± 108 nM) and GCAP2 ($K_D = 418$ nM ± 135 nM), which are similar values to those determined in the absence of Ca\(^{2+}\). These results indicate that the interaction of GCAPs with GC-E is Ca\(^{2+}\)-independent, confirming previous enzyme activation studies. The phase changes observed in the presence of Ca\(^{2+}\) (Fig. 2d) are significantly smaller than those observed in the absence of Ca\(^{2+}\) (Fig. 2a), further supporting the Ca\(^{2+}\)-independent binding of GCAPs to GC-E.
in Ca^{2+}-free solution. GCAP2 did show a slightly higher affinity for human GC-E in the apo- and Ca^{2+}-bound state than did the GCAP1 species, but within experimental error both of the K_D-values were found to be in the submicromolar range. By using the BSI assay approach, it was possible to confirm previous circumstantial evidence reporting Ca^{2+}-independent interaction of GCAPs with mammalian GCs. Furthermore, we showed that the binding process is of medium affinity, which is similar to previous reports of EC_{50}-values. Concentrations of GCAPs, at which the activation of GCs is halfmaximal are expressed as EC_{50}-values and are interpreted as apparent affinity constants.

The validity of these observations were confirmed by excluding impairment functionality of human GC-E in HEK vesicles, by performing a standard GC-Assay and confirming enzymatic functionality (data not shown). In this case, HEK cell membranes expressing human GC-E were processed using sonication to form vesicles. Reference recordings were made by BSI with HEK cell vesicles lacking human GC-E. Additionally we used calmodulin (CaM), another member of the EF-hand superfamily of Ca^{2+}-binding proteins, which is very similar to NCS proteins in three dimensional folding, to test whether the BSI binding signal is specific for GCAPs. The result of this binding assay is depicted in Fig. 2, showing that titration of GC-E with increasing concentration of calmodulin resulted in a phase shift below the limit of detection for binding. This result further confirms that the BSI signal is quantifying specific binding of the GCAPs to human GC-E (Fig. 2).

The juxta-membrane and kinase homology domain exclusively interacts with GCAP1. Having proven the suitability of the BSI assay system for the GC/GCAP system we set out to critically test opposing opinions about the localization site of GCAP1 and GCAP2 interaction domains. This task was uniquely enabled by using BSI to study the molecular interactions of intracellular parts of human GC-E. Here we divided GC-E into two fragments, one comprising the amino acid residues M496 to K806 corresponding to the juxta-membrane and kinase homology domain (JMD + KHD). The other fragment corresponded to the catalytic domain (CD) found in the region from G868-S1103. Both of these fragments were fused to a solubility enhancer, maltose binding protein (MBP) to ensure they were soluble. Either GCAP1 or GCAP2 would bind to both fragments or both GCAPs would target to different regions. The topology of a G-E dimer is shown in Fig. 3 indicating protein domains and the two alternative scenarios of GCAP interaction that are currently discussed.

Figure 4a presents the results from the BSI assay performed with GCAP1 and GCAP2 on the JMD-KHD fragment. Importantly, binding of GCAP1 to the JMD-KHD fragment was observed, with a K_D-value of 841 nM ± 134 nM, while GCAP2 showed only a minor BSI signal (phase shift). The lower curve in Fig. 4 clearly indicates this signal is not of the magnitude to be considered a quantifiable binding event. Further, since all reference measurements were performed with the exact same concentration of MBP and subtracted from the BSI binding signal we can exclude a possible interaction of the fusion MBP part with the GCAPs. Taking into account
that saturating Ca\(^{2+}\) was present in the measuring buffer, which might have affected the binding of GCAP2 to the JMD + KHD fragment, we reproduced the same BSI assay except 1 mM EGTA was present (data not shown). The Ca\(^{2+}\)-bound state of either GCAP1 or GCAP2 had virtually no influence on the interaction with the JMD-KHD fragment. To further validate our observations we used isothermal titration calorimetry (ITC) and titrated either GCAP1 or GCAP2 with the JMD\(^{+}\)KHD fragment at 25 °C. Using a one binding site model implemented in the ITC evaluation software (Origin 4.0, MicroCal) we determined an affinity constant for GCAP1 binding that is consistent with the BSI data ($K_D = 580 \, \text{nM} \pm 250 \, \text{nM}$) (data not shown). The stoichiometry of the binding shows a one to one binding for GCAP1 to the GC-fragment ($n = 0.9 \pm 0.2$). However, we were only able to record two ITC runs of sufficient quality, four different runs showed no detectable enthalpy change. We attribute these findings to the inherent instability of the JMD\(^{+}\)KHD fragment and consider the BSI assay as advantageous over ITC investigating for targets, which are intractable. In the experimental set-up of our ITC measurements, the JMD\(^{+}\)KHD fragment had to be placed in the measuring chamber for several hours at 25 °C, whereas for the BSI assay the JMD + KHD sample is kept on ice shortly before the injection started.

Results from the binding experiments for the GCAPs to the CD fragment are more difficult to interpret: amplitudes of GCAP2 binding titration showed a dip at very low GCAP2 concentrations, but then displayed increasing amplitudes saturating at 40 mRad (Fig. 4b). Binding signals of GCAP1 were lower and data showed higher scatter or poorer reproducibility (Fig. 4c). These data are consistent with previous reports showing interaction of GCAP2 with the CD domain and interaction of GCAP1 with lower affinity\(^{15}\). However, we can only speculate where the initial BSI signal decrease upon GCAP2 titration to the CD-fragment came from. Membrane

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**Figure 4.** Interaction of GCAP1 and GCAP2 with fragments of human GC-E determined by BSI in the presence of 200 \(\mu\)M Ca\(^{2+}\). (a) Interaction of GCAP1 and GCAP2 was tested with the JMD + KHD fragment. An Interaction of GCAP2 with this fragment was not observed, for GCAP1 the fitting of BSI data gave $K_D = 841 \, \text{nM} \pm 134 \, \text{nM}$ ($n = 3$). (b,c) Interaction of GCAP2 and GCAP1 with the CD fragment of human GC-E ($n = 4$).
bound GCs, either hormone ligand receptors or sensory GCs form dimers as functional units. Adding GCAP2 to the CD-fragments might have induced a dimerization of the fragment, which is reflected in the negative BSI signal relative to observations reported above. This observation would be consistent with a previous report showing that the presence of GCAPs facilitates formation of dimers in photoreceptor GC-E and with a recent report on the BSI signaling mechanism that shows certain interactions can produce a negative-going binding curve.

**GCAPs do not share a single binding site.** Recently Peshenko et al. identified a crucial amino acid residue (M823) located in the dimerization domain of human GC-E involved in GCAP binding by a co-localization approach. Further the same group suggested a model in which GCAP1 and GCAP2 share a single binding site located in a region spanning the juxta-membrane domain to the dimerization domain, shown by a comprehensive study employing chimeras of human GC-E and its co-localization with GCAPs. Such a scenario, in which GCAP1 and GCAP2 interact with GC-E in a mutually exclusive mode upon fluctuating Ca2+-levels, is at odds with our findings here and with several other reports. We quantified affinities for GCAPs by BSI in the sub-micromolar range, which were negligibly affected by the Ca2+-bound state of GCAPs. Bovine rod outer segments contain a GCAP1 and GCAP2 concentration of about 3 μM, which would result in the GC-E being saturated, assuming Kd values determined in this study. However, the medium affinity binding of GCAPs to full-length GC-E could also reflect a dynamic complex formation and dissociation, which occurs in the absence and presence of Ca2+. The GC-E/GCAP complex might undergo conformational rearrangements that could be the reason why different experimental approaches (peptide competition, mutagenesis and crosslinking in combination with mass spectrometry) gave different, in some cases inconsistent results.

**Conclusion**
The BSI system was used in the last decade in protein interaction studies and, the clear Ca2+-dependent interaction of recoverin with its target GRK1 represents a system in which we can induce binding simply by adding low concentrations of Ca2+, clearly showing that a BSI binding signal is specific. The signal occurs only in the presence of Ca2+, whereas no binding signal was recorded in the absence of Ca2+. BSI enabled the resolution of contradicting findings regarding GCAP interaction sites and we are able to report that GCAP1 and GCAP2 do not share a common binding site. Planned investigations for future studies beyond the scope of this work will address the investigation of more complex BSI response patterns. These might result from a shift in protein monomer-dimer equilibria producing negative going signals.

**Methods**

**Molecular cloning of GC-fragments.** The cloning of full length human GC-E into a pIRE2-eGFP vector (Clontech) for the expression in mammalian cell culture was described earlier.

Without a fusion part we were not able to obtain any soluble fragment from overexpression in *E. coli*, due to the strong tendency of constructs to aggregate. Hence, we fused the respective fragments to the solubility enhancer maltose binding protein (MBP), which allowed to obtain a soluble fragment after dialytic refolding from the inclusion bodies. Fusion proteins were designed accordingly and named MBP-KHD and MBP-CD. The MBP-KHD is a fusion protein with maltose binding protein (MBP) attached to a human GC-E fragment corresponding to the juxta-membrane and kinase homology domain (MD-KHD, M496–K806). MBP-CD corresponds to the catalytic domain (CD, G868–S1103) of the human GC-E. The coding sequence of KHD was amplified on wildtype human GC-E sequence by PCR adding a BamHI and a partial NcoI site using the primers 5’-GTCTCCGGGCCCCACAAAG-3’ and 5’-AAAGGATCCACTGGACCGGACTGGAAGGTGTA-3’. The M11-MBP vector (provided by the EMBL, Heidelberg, Germany) was cut by NcoI, treated by Klenow fragment to obtain a polynucleotide kinase and afterwards cut by BamHI. The PCR fragment was cut by BamHI, phosphorylated with T4 Polynucleotide Kinase and ligated into the M11-MBP vector using spectrophoresis protocols. MBP-CD was obtained by PCR on wildtype sequence by PCR adding restriction sites BamHI and NcoI using the primers 5’-AAAGGATCCACTGGACCGGACTGGAAGGTGTA-3’ and 5’-GATGATTCAAGGACCTGAGCCGGGCGGCGGCG-3’ and ligated into M11-MBP vector (EMBL) following standard protocols. The fragments were verified by DNA sequencing (GATC, Germany).

**Protein expression and purification.** MBP constructs were expressed in *E. coli* BL21 (+) cells. For this *E. coli* cells were transformed with the respective plasmid and grown to an OD600 = 0.6. Expression was induced by 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside), after 4 h the cells were harvested at 5000 × g for 15 min and the pellet was resuspended in 20 mM HEPES KOH pH: 7.4, 1 mM DTT, 1 mM PMSF, 5 U/mL DNAse and lysed by sonification on ice. To separate insoluble from soluble proteins the lyzed cells were centrifuged at 50,000 × g for 1.5 h at 4 °C. The MBP constructs were exclusively present in the insoluble inclusion bodies verified by SDS-PAGE. To extract the constructs the insoluble pellet was washed twice with 20 mM HEPES/KOH pH 7.4 and homogenized in 30 mL 8 M Urea and incubated overnight at 4 °C. After centrifugation at 50,000 × g for 30 min at 4 °C, the MBP construct containing supernatant was dialyzed twice against 3 L of 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM DTT for at least 4 h and centrifuged again at 50,000 × g for 30 min at 4 °C. To isolate the respective MBP construct the supernatant was loaded onto approximately 10 mL of amylase resin (NEB) in a self-packed gravity flow column at 4 °C. After washing with 10 column volume 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM DTT the MBP constructs were eluted using the same buffer supplemented with 10 mM maltose. The buffer was exchanged to 50 mM (NH4)HCO3 and protein sample were lyophilized and stored at −80 °C until further use.

For reference measurements MBP was obtained by cleaving the respective GC fragments with TEV-Protease. For this, the MBP-GC construct was dissolved in 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM DTT and TEV protease was added in a ratio of 1:10 and incubated overnight at 4 °C. The purification of the MBP fragment was...
done exactly as described for the MBP-GC constructs, except that subsequently a size exclusion chromatography was performed using 20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM EDTA and a HiLoad 26/60 Superdex 75 prep gradient gel filtration column (GE Healthcare).

GCAP1 and GCAP2 was expressed in E. coli and purified by a combination of size-exclusion chromatography and anion-exchange chromatography exactly as described before. To obtain myristoylated GCAPs E. coli cells were co-transformed with yeast N-terminal myristoyl transferase (kindly provided by Dr. Jeffrey Gordon, Washington University School of Medicine, St. Louis, USA) and the LB medium was supplemented with myristic acid. Myristoylated Recoverin and the N-terminal GRK1 fragments (NRK) was prepared exactly as described earlier.

Human GC-E was expressed in HEK-Flp 293 cells as described previously. Briefly, HEK cells were cultured in minimal essential medium to 80% confluency and harvested in 5 mM KCl, 20 mM MgCl₂, and 150 mM phosphate buffer (3–5 × 10⁵ cells). 5 μg Vector DNA (pRES2-eGFP, Clontech) containing the cDNA for human GC-E was added and electroporated with the CLB system (Lonza). After 24 h the medium was exchanged and supplemented with G418 (Merck Millipore), a selective antibiotic to generate a stable cell line expressing human GC-E. Single colonies of HEK cells were picked and checked by western blot for the expression of human GC-E. All experiments were done using a stable cell line originating from a single cell. For the experiments the stable cell line was cultivated up to a confluency up to 80% and harvested (500xg, 5 min) and washed twice with ice-cold PBS to remove medium residues. All cell pellets were frozen in liquid nitrogen and stored at −80 °C until further use.

**Isothermal titration calorimetry.** Isothermal titration calorimetry (ITC) was performed using a VP-ITC instrument from MicroCal (Northampton, MA, USA) at T = 25 °C. Purified MBF fusion protein was present in the recording cell in the exact same buffer used for the BSI assays (30 mM Mops/KOH pH 7.2, 60 mM KCl, 4 mM NaCl, 1 mM DTT, 3.5 mM MgCl₂, 1 mM GTP, 0.3 mM ATP) at 0.5 μM and titrated with myristolated GCAP1. All protein samples were dissolved in filtered (0.22 μm) and degassed titration buffer and sonicated at 0 °C for 5 min. Afterwards the protein samples were ultracentrifuged (100,000 g, 15 min) and the supernatant was carefully withdrawn for the subsequent ITC experiments.

GCAP was placed in the microcylinder chamber at 10 μM and the titration was performed by subsequent injections of 5 μl GCAP solution into the recording cell keeping a time interval of 180 s between injections and an initial delay of 600 s after temperature equilibration. Control injections of GCAP into MBP, lacking a GC-E fusion part were also performed accordingly and subtracted from the sample titration. The data was fitted using a one ligand binding model employing the Microcal software (Origin) to estimate a binding constant (Kₜₐₜ) at the given temperature.

**BSI instrumentation.** BSI instrumentation has been described in detail previously. Briefly, the instrument is comprised of a linear polarized helium–neon laser (2 mW, Melles Griot) a microfluidic chip (Micronite, Netherlands), and a charge-coupled device (CCD) camera (CCD-S3600-D, Alphalas, Germany). The respective sample is introduced into the microfluidic chip, which is designed to create a resonance cavity with a long effective path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length. The incident coherent light is converted into an interferometric fringe pattern, which is captured by the CCD camera. Fourier analysis is used to determine the phase change (in radians) to quantify spatial refractive path length.

**Protein interaction assay using BSI.** The interaction studies were performed using either full-length human GC-E or fragments of human GC-E expressed as MBP fusion proteins. For the BSI interaction studies with full-length human GC-E, HEK vesicles expressing GC-E were prepared as follows. Approximately 4 × 10⁶ cells were resuspended in titration buffer (30 mM Mops/KOH pH 7.2, 60 mM KCl, 4 mM NaCl, 1 mM DTT, 3.5 mM MgCl₂, 1 mM GTP, 0.3 mM ATP) supplemented with 1 mM PMSF and probe sonicated for 2 min. The particle size of the resulting HEK cell vesicles was determined by dynamic light scattering to range around 50 nm. Afterwards the total protein amount was determined using a standard Bradford assay and the HEK vesicle particle size of the resulting HEK cell vesicles was determined by dynamic light scattering to range around 50 nm.

For the assay of recoverin and GRK1 we used buffer condition (20 mM HEPES/KOH pH 7.5, 100 mM NaCl, 1 mM MgCl₂, 200 μM CaCl₂, or 1 mM EGTA) to match previously SPR studies employing this system. Since the N-terminal GRK1 fragment has a GST fusion part, we used GST alone in a reference dilution series.

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