Directly light-regulated binding of RGS-LOV photoreceptors to anionic membrane phospholipids

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Edited by Winslow R. Briggs, Carnegie Institution for Science, Stanford, CA, and approved July 6, 2018 (received for review February 15, 2018)

We report natural light–oxygen–voltage (LOV) photoreceptors with a blue light-switched, high-affinity ($K_D \sim 10^{-10}$ M), and direct electrostatic interaction with anionic phospholipids. Membrane localization of one such photoreceptor, BcLOV4 from \textit{Botrytis cinerea}, is directly coupled to its flavin photocycle, and is mediated by a polybasic amphipathic helix in the linker region between the LOV sensor and its C-terminal domain of unknown function (DUF), as revealed through a combination of bioinformatics, computational protein modeling, structure–function studies, and optogenetic assays in yeast and mammalian cell line expression systems. In model systems, BcLOV4 rapidly translocates from the cytosol to plasma membrane (-1 second). The reversible electrostatic interaction is nonselective among anionic phospholipids, exhibiting binding strengths dependent on the total anionic content of the membrane without preference for a specific headgroup. The in vitro and cellular responses were also observed with a BcLOV4 homolog and thus are likely to be general across the dikarya LOV class, whose members are associated with regulator of G-protein signaling (RGS) domains. Natural photoreceptors are not previously known to directly associate with membrane phospholipids in a light-dependent manner, and thus this work establishes both a photosensory signal transmission mode and a single-component optogenetic tool with rapid membrane localization kinetics that approaches the diffusion limit.

Photoreceptors are complex protein machines that transduce optical sensory inputs into diverse outputs in signaling and energy conversion. Establishing the structure–function relationship and signaling modes of novel photosensory proteins enhances understanding of organismal adaptation to a universal cue of light. Among the known photoreceptors, light–oxygen–voltage (LOV) proteins (1–3) comprise the most ubiquitous and topologically diverse class. LOV photosensory signaling, which relies on a flavin chromophore bound within a Per–Amt–Sim (PAS)-type sensory domain, is initiated by blue light-induced formation of a reversible covalent cysteiny1-flavin photoadduct. The subsequent structural changes in the surrounding protein alter the activity of effector domains and peptides fused up/downstream to the sensor domain itself, controlling biological function (1–6).

One way the diversity of LOV-regulated biochemical activities has been revealed is through the use of large-scale genomic analyses, including a recent study of ours that cataloged over 6,700 LOV domains with over 100 combinatorial sensor–effector arrangements (5). This topological flexibility confers great functional diversity in the ways signals are transmitted as well as the kinds of biological responses that are controlled by these photoreceptors (2–5, 7). Beyond their fundamental importance in photobiology, novel LOV proteins provide valuable components and molecular engineering principles for creating optogenetic tools to perturb the physiology of targeted cells. For example, bacterial LOV proteins have been adapted for light-regulated transcriptional activation (8) and adenylate cyclase activity (9) in eukaryotes. Likewise, truncated LOV sensor domains with established signaling structure–function offer numerous approaches to engineering chimeric proteins to confer optically inducible functions to a wide range of fusion partners (10–15).

Although the breadth of LOV function can be inferred from bioinformatics, reported protein-level biochemical characterization and structure–function analyses of novel-effector LOVs are still rare. As part of a broad survey of LOV proteins (3), we previously identified one such new class of dikarya fungal LOVs associated with regulator of G-protein signaling (RGS) domains (Fig. 1 A and B and SI Appendix, Fig. S1). RGS proteins are the primary fast terminators of G-protein–coupled receptor (GPCR) signaling, serving as GTPase accelerating proteins (GAPs) on activated G-protein partners (14–16). While other bioinformatics and cell biology studies have predicted the presence of RGS-LOV proteins (17–21), direct experimental evidence of photosensory function has not been shown. Their genetic deletion does not beget any pronounced phenotypic change (17), and their transcriptional levels are not under light-dependent transcriptional regulation (22). Thus, protein-level photochemical characterization is required to confirm bona fide photosensory activity for this class of LOV proteins.

Significance

Light–oxygen–voltage (LOV) domain photoreceptors are found ubiquitously in nature and possess highly diverse signaling roles and mechanisms. Here, we show that a class of fungal LOV proteins dynamically associates with anionic plasma membrane phospholipids by a blue light-switched electrostatic interaction. This reversible association is rapidly triggered by blue light and ceases within seconds when illumination ceases. Within the native host, we predict that these proteins regulate G-protein signaling by the controlled recruitment of fused regulator of G-protein signaling (RGS) domains; in applied contexts, we anticipate that engineered chimeric versions of such proteins will be useful for rapid optogenetic membrane localization of fused proteins through direct interaction with the membrane itself, without requiring additional components to direct subcellular localization.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The plasmid for mammalian codon-optimized BcLOV4 fused to mCherry and the plasmid for native sequence BcLOV4 fused to mCherry were deposited with AddGene (https://www.addgene.org) (accession nos. 114595 and 114596).

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This article contains supporting information online at www.pnas.org/cgi/doi/10.1073/pnas.1802832115/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1802832115
Here, we report that these cytosolic LOV proteins dynamically and reversibly associate with the plasma membrane by directly light-regulated and high-affinity binding to anionic phospholipids, as revealed through a combination of bioinformatics, computational protein modeling in Rosetta, in vitro structure–function studies with purified recombinantly expressed protein, and optogenetic assays in multiple eukaryotic heterologous expression systems. The photosensory phenomenon was found to be directly coupled to flavin photocycling and is likely general across RGS-LOVs. This study establishes a significant signaling mechanism relevant to natural photoreceptors, and broadly applicable to single-component optogenetic tools for dynamic membrane localization.

Rapid Membrane Localization in Cells in Response to Blue Light. Light-activated aggregation has not previously been reported for LOV proteins, although oligomerization into photobodies is known among natural phytochromes, cryptochromes, and their engineered optogenetic variants (24, 25). Thus, to functionally probe whether BcLOV4 forms photobodies in cells, BcLOV4 was visualized by fluorescence microscopy when heterologously expressed in mammalian cells (Fig. 2), which we used because B. cinerea is pathogenic and less genetically tractable than HEK cells. Cells expressing 3×-FLAG-tagged BcLOV4 were fixed in blue light or the dark, and then stained with fluorescent dye-labeled anti-FLAG monoclonal antibody. To our surprise, BcLOV4 did not primarily form photobodies in cells but instead localized to the plasma membrane in a blue-light-dependent manner (Fig. 24). Both the dynamic membrane localization in cells and in vitro photoggregation were also observed with the homolog from the black yeast C. glabrata (hereon called CeRGS) (GenBank accession number E7N3699.1) (SI Appendix, Fig. S5). Thus, importantly, the in vitro and cellular phenomena are likely general to the class of RGS-LOV proteins. Due to low purified recombinant protein yield and heterologous expression levels of CeRGS in cellular assays, data reported hereafter focus on BcLOV4.

To determine the dynamics of this translocation process, mCherry-tagged BcLOV4 variants were directly visualized, using cotransfected isoprenylated GFP as a plasma membrane marker in HEK cells. BcLOV4 had an optical absorbance peak at \( \lambda_{\text{max}} = 450 \text{ nm} \) with triplet-peak fine structure (Fig. 1C) indicative of a LOV-bound flavin mononucleotide (FMN) cofactor (see SI Appendix, Fig. S2, for cofactor isolation). BcLOV4 photocycled with rapid thermal reversion kinetics (\( \tau_{\text{off}} = 18.5 \pm 2.0 \text{ s} \)) (Fig. 1D). However, stable photocycle measurements required in vitro stabilization by high salinity alone (0.5–1 M NaCl) or in combination with glycerol (10%), or immobilization on solid-phase supports (see SI Appendix, Fig. S3, for photocycling summary). In the absence of such stabilization, illuminated BcLOV4 quickly aggregated into turbid solutions of micrometer-scale colloids as measured by dynamic light scattering (DLS) (Fig. 1E). This in vitro photoaggregation was preventable with high stabilization (e.g., 1 M NaCl), reversible with intermediate stabilization (e.g., 0.5 M NaCl); and irreversible in normal-salinity PBS, eventually precipitating from solution (SI Appendix, Fig. S4). This phenomenon was dependent on flavin photocycling, since it was abolished for a photochemically inactive C292A BcLOV4 mutant. This mutant is still a holoprotein, but the C292A mutation prevents the formation of the critical cysteiny1-flavin photodadduct (23) that initiates canonical LOV signaling, such that it mimics a permanently dark-adapted protein even in the presence of blue light.
The functional kinetics of membrane association and undocking measured by live-cell imaging were fast (HEK $\tau_{on} = 1.11 \text{ s}$, $\tau_{off} = 89.1 \text{ s}$) (Fig. 2 B–F). The membrane association was on the timescale of diffusion to the inner leaflet (Fig. 2 C and E) ($\sim 0.7–1.6 \text{ s}$; see Materials and Methods for timescale estimate determination), and such kinetics is indicative of a high-affinity interaction between BcLOV4 and its membrane target. We hypothesized that such a light-switched interaction could occur directly between BcLOV4 and membrane lipids, because the photosensory signal-transmitting $\alpha$-helix linker is fused to a polybasic amphipathic helix (AH1 in Fig. 1 A) similar to those involved in membrane association. Thus, consistent with pmGFP cellular kinetics, BcLOV4 indeed bound anionic lipids but not zwitterionic bilayers. Therefore, the presence of other proteins. The measurements were made with the photochemically inactive C292A and constitutively active Q355N mutants, since controlled illumination within the instrument was not possible. BcLOV4-mCherry variants were used for SPR assays both to maintain consistency with droplet assays, and for improved solubility and protein yield. The BcLOV4 constitutively active mutant affinity for 20% PS liposomal bilayers was $K_d^{C292A} = 130 \text{ nM}$, or $\sim 20$-fold enhanced vs. the photochemically inactive mutant $K_d^{Q355N} = 3.2 \mu\text{M}$ (Fig. 3E). Thus, consistent with biophysical inferences from cellular kinetics, BcLOV4 indeed associated with anionic lipids and undocked from zwitterionic bilayers.

**Directly Light-Regulated and High-Affinity Interaction with Anionic Phospholipids.** Initial protein–lipid overlay screening assays suggested that BcLOV4 bound anionic lipids but not zwitterionic ones, but this assay tests for headgroup interactions without recapitulating a membrane interface. Thus, to test for this new direct protein–lipid interaction with a more realistic membrane target (Fig. 3), we created droplets of water-in-oil (w/o) emulsions (29) containing purified recombinant BcLOV4-mCherry in the dispersed/aqueous phase, and phospholipid monolayers at the droplet interface to emulate the plasma membrane inner leaflet (Fig. 3D). Artificial membranes were composed of the zwitterionic phosphatidylcholine (PC) mixed with anionic phospholipids of varying concentration and headgroup charge density.

These droplet assays allowed for complete control over illumination conditions and membrane compositions without complications introduced by the presence of other proteins. The facile customization and ability to multiplex on an automated fluorescence microscope made the system highly useful for screening and cross-validating other methods for establishing binding interactions, like surface plasmon resonance (SPR). As seen in 20% phosphatidylserine (PS)-containing emulsions of similar PS composition to mammalian membranes (Fig. 3 B and C), illuminated BcLOV4-mCherry primarily localized to the phospholipid interface, instead of aggregating as observed in lipid-free bulk solution experiments. Conversely, BcLOV4 formed colloids in lieu of binding pure zwitterionic PC interfaces with only positively charged headgroups. Both light-activated localization and aggregation were observed in the aqueous compartment diminished as salinity increased (SI Appendix, Fig. S6), suggesting an electrostatic basis for these phenomena. Localization was diminished in blue light for the photochemically inactive C292A mutant (23), confirming direct coupling of the phenomenon to signaling initiation by flavin photocycling as opposed to an unknown blue light interaction (Fig. 3D). Conversely, localization was persistent in the absence of illumination with a constitutively active Q355N mutant that structurally mimics the signal-transducing conformation of the LOV $\alpha$-helix linker region as if it were in a permanently lit or active signaling state, even in the absence of illumination (30–32) (Fig. 3D). The constitutively active mutant also retained the BcLOV4 binding preference of light-activated wild-type BcLOV4 for net anionic lipids over purely zwitterionic PC interfaces. Thus, optical activation of the interaction with membrane phospholipid is consistent with known structure–function determinants of LOV signaling with respect to flavin photocycling and signal transmission via the $\alpha$-helix.

Dissociation constants for BcLOV4 binding to immobilized liposomal bilayers were next measured by SPR. The measurements were made with the photochemically inactive C292A and constitutively active Q355N mutants, since controlled illumination within the instrument was not possible. BcLOV4-mCherry variants were used for SPR assays both to maintain consistency with droplet assays, and for improved solubility and protein yield. The BcLOV4 constitutively active mutant affinity for 20% PS liposomal bilayers was $K_d^{Q355N} = 130 \text{ nM}$, or $\sim 20$-fold enhanced vs. the photochemically inactive mutant $K_d^{C292A} = 3.2 \mu\text{M}$ (Fig. 3E). Thus, consistent with biophysical inferences from cellular kinetics, BcLOV4 indeed associated with anionic lipids and undocked from zwitterionic bilayers.
possesses a high-affinity light-switched interaction with anionic phospholipids. Binding increased with total anionic content (with PS, Fig. 3F), but there were minimal differences between phospholipids of different headgroup charge density under conditions of matching total charge (Fig. 3G). Thus, BcLOV4 membrane binding is charge dependent but nonspecific to headgroup identity, unlike the well-established preference of pleckstrin homology (PH) domains for certain phosphatidylinositol phosphates (PIPs) (33), or similar lactadherin-C2 domain specificity for PS (34).

**Key Structure–Function of the Dynamic Protein–Lipid Interaction.** Having identified the light-switched interaction partner as an anionic phospholipid, we next sought to determine the protein binding site (Fig. 4) and focused on the polybasic amphipathic helix in the linker region that is largely conserved among the fungal homologs (AH1) (Fig. 4A). In BcLOV4, this helix possesses a conserved “FFK” motif (residues 412–414) found in membrane-interacting amphipathic helices of Bcl-2-associated death promoter (BAD) (35), kinase suppressor of RAS (KSR) (36), and cecropin anti-microbial peptides (37), and a “FFK” sequence (residues 408–410) found at the membrane interface of the M2 proton channel of influenza A [Protein Data Bank ID code 2rfl]. In such motifs, aromatic side chains putatively insert into the phospholipid bilayer, while the proximal lysine side chains electrostatically bind anionic lipids enriched in the inner leaflet without great headgroup specificity (27) (Fig. 4B).

Thus, candidate phenylalanine and tyrosine residues within this region were mutated to alanines (Fig. 4C–E). The BcLOV4-AH1 mutant (amphipathic helix mutant) photocycled similarly to wild-type protein (SI Appendix, Fig. S3). In SPR assays, the BcLOV4-AH1 constitutively active mutant showed a 10-fold reduction in affinity, $K_{d,\text{const}} = 130 \pm 75 \text{ nM}$ and 20-fold enhanced over the photochemically inactive mutant ($K_{d,\text{const}} = 3.2 \pm 1.2 \text{ nM}$). (i) The 0–20 μM range, with fit only for constitutively active mutant for clarity, and (ii) 0–2 μM range. $n = 2$–7; error, SEM. (F) SPR measures of constitutively active mutant binding to mixed POPC liposomes of varying total anionic charge density. $n = 3$; error, SD. (G) SPR binding assessments of constitutively active mutant to lipids of different headgroup charge density, in liposomes of matching total anionic charge density of 20% ($n = 3$; error, SD). (F and G) CL, cardiolipin; PC, phosphatidylcholine; PIP2, phosphatidylinositol(4,5)-biphosphate; PIP3, phosphatidylinositol(3,4,5)-triphosphate; PS, phosphatidylserine.

**Fig. 3.** In vitro binding to anionic membrane lipids. (A) Schematic of BcLOV4 in lipid-stabilized w/o emulsions. (B) Fluorescence micrographs of wild-type BcLOV4 fused to mCherry. Translocation to the inner leaflet-like interface is observed with increasing anionic PS content, but not with purely zwitterionic PC interfaces. (C) Phospholipid interface binding curves, calculated as the membrane interface:dispersed phase ratio (normalized) of BcLOV4 in the light and dark. $n = 20$–75 droplets; error, SEM. (D) Constitutively active BcLOV4 Q355N structurally mimics the photoactivated signaling state, is localized to the interface in the dark, and retains its preference for net anionic phospholipids over zwitterionic ones. The photochemically inactive C292A mutant cannot form a covalent cysteinyl-flavin photoadduct and remains in the aqueous dispersed phase even upon illumination. (E–D) Blue light pulses: $\lambda = 440\pm20 \text{ nm}, 5 \times 15 \text{ mWcm}^{-2}$. mCherry imaging: $z_{\text{ex}} = 550/15 \text{ nm}, z_{\text{em}} = 630/75 \text{ nm}$. (Scale bar: 25 μm.) (E) Affinity measures by SPR to 80% PC/20% PS mixed liposomal bilayers. The interaction with constitutively active BcLOV4 is high affinity ($K_{d,\text{const}} = 130 \pm 75 \text{ nM}$) and 20-fold enhanced over the photochemically inactive mutant ($K_{d,\text{const}} = 3.2 \pm 1.2 \text{ nM}$). (i) The 0–20 μM range, with fit only for constitutively active mutant for clarity, and (ii) 0–2 μM range. $n = 2$–7; error, SEM. (F) SPR measures of constitutively active mutant binding to mixed POPC liposomes of varying total anionic charge density. $n = 3$; error, SD. (G) SPR binding assessments of constitutively active mutant to lipids of different headgroup charge density, in liposomes of matching total anionic charge density of 20% ($n = 3$; error, SD). (F and G) CL, cardiolipin; PC, phosphatidylcholine; PIP2, phosphatidylinositol(4,5)-biphosphate; PIP3, phosphatidylinositol(3,4,5)-triphosphate; PS, phosphatidylserine.
by light-induced structural rearrangements that expose a critical polybasic amphipathic helix at the LOV-DUF linker that is inhibited by the RGS domain in the dark (Fig. 4f).

Blue Light-Dependent Membrane Localization in Fungus. To determine whether the fungal-derived BcLOV4 associates with membranes in a blue light-inducible manner in fungus, dynamic localization assays were performed in Saccharomyces cerevisiae yeast (Fig. 5). Such confirmation of the photosensory response in fungal cells is possible that the photosensory response in fungal cells is confirmed by blue light-induced structural rearrangements that expose a critical polybasic amphipathic helix at the LOV-DUF linker that is inhibited by the RGS domain in the dark (Fig. 4f).

Discussion

Our cumulative findings suggest a photosensory signal transmission mode by RGS-LOV of rapidly blue light-inducible, and reversible, membrane association mediated by electrostatic interactions with anionic phospholipids. While other membrane-binding proteins contain PAS domain sensors (41, 42) related to LOV domains, such as PhoQ, Aer, and LuxO (43, 44), these are ligand-regulated transmembrane proteins unlike the cytoplasmic photoreceptors reported here. We underscore that this signaling mode was not anticipated from hidden Markov model-based bioinformatics searches for conserved domains, which found nothing C-terminal to the LOV sensor. De novo secondary-structure and Rosetta (45) structural predictions suggest that the DUF in this region may adopt a PAS-like fold with antiparallel β-sheets (SI Appendix, Fig. S7D), and thus it is possible that the LOV–DUF interaction is an evolutionarily conserved PAS/PAS interaction as observed in other systems. It should be noted that other lipid interaction sites may exist beyond the critical amphipathic helix between the LOV and DUF domains. A future high-resolution structure of the lipid-bound state will greatly inform the proposed biophysical model, as well as conclusively determine whether the DUF is indeed a PAS domain.

Membrane localization is a known prerequisite for the canonical GAP activity of RGS proteins (46, 47). Therefore, it is plausible that the signal transmission mode proposed in Fig. 4f—and the photosensory response that was consistently observed across in vitro and cellular systems (including in yeast) and across different RGS-LOV proteins (BcLOV4 and CeRGS)—serves to regulate
interactions with cognate Gα proteins at the membrane in-host. Little is known about the photobiological role of BcLOV4 (18), its interactions with the three Gα proteins of Botrytis (48), and the physiological roles of the latter. However, the isolated (or truncated) RGS domain of MoRGS5 (GenBank accession number EHA46884.1), the RGS-LOV of Magnaporthe oryzae (rice blast fungus), does interact in yeast two-hybrid assays with its cognate Gα protein, MagB (17), which is involved with hydrophobic sensing and plant infectivity (49, 50). Genetic knockout of MoRGS5 (ΔMoRGS5) results in increased intracellular cAMP levels, further implicating a role for RGS-LOV proteins in cell signaling (17, 18). ΔMoRGS5 strains, however, exhibit no pronounced organism-level phenotypic difference from wild-type strains. Thus, while a conclusive photobiological role has yet to be established for RGS-LOV proteins (51), which of note have not been shown to be photochemically active to date, the findings here provide a potential biophysical mechanism by which they may affect fungal physiology: through light-regulated and reversible membrane association of a Gα-interacting photosensory to fine-tune Gα-dependent cAMP signaling (18).

In an applied context, BcLOV4 also contributes a useful single-component optogenetic system for photoinducible membrane localization that is compatible in yeast and mammalian expression systems. Its translocation kinetics was apparently limited by diffusion in a cellular context and thus approaches a practical limit for rapid optogenetic membrane localization. Unlike the indirect membrane binding of optogenetic tools that rely on heterodimerization between cytosolic and membrane-bound partners (52, 53), BcLOV4 as a single-component system is insensitive to heterogeneity in relative expression level tuning of two components, and is more facile in transgene delivery.

A common goal in optogenetics, and a common motivation for establishing fundamental structure–function that may beget new function studies at large.

Materials and Methods

Genetic Constructs and Protein Expression.

**Bacterial genetic constructs.** For protein expression, genes fragments encoding for BcLOV4 (GenBank accession number CDD5251.1), Cyphellophora europea LOV (ETN36999.1), and Exophila dermatitis LOV (EH6Y0539.1) were ordered from Integrated DNA Technologies as gBlocks and assembled by Gibson cloning or PCR assembly. Transgenes were cloned into a pET21/28-derivated bacterial expression vector. C-terminal mCherry fusions with a (GGGSS), linker were generated by Gibson cloning. Genetic constructs were transformed into competent Escherichia coli (C2984H; NEB Turbo). Mutants were generated by QuikChange site-directed mutagenesis. All sequences were verified by Sangen sequencing.

**Mammalian genetic constructs.** DNA sequence of BcLOV4 was human codon-optimized (Genscript). The C-terminal mCherry fusion was created as described above. The mCherry-free variant with a C-terminal “3xFLAG” tag (Sigma Aldrich) had a G4GSS linker. Transgenes were cloned into the pcDNA3.1 mammalian expression vector (Invitrogen).

**Yeast genetic constructs.** BcLOV4-mCherry was cloned into a pRSIII26 yeast expression vector with uracil auxotrophic marker (plasmid #35469; Addgene) (62), and transformed into S. cerevisiae (ATCC 203188 strain BY4741) competent cells prepared using a Y2hmo Research Frozen-EZ Yeast Transformation II Kit. Cells were cultured in uracil dropout medium (Sigma-Aldrich).

**Recombinant protein expression, isolation, and purification.** Recombinant proteins were expressed in E. coli BL21(DE3). Cells were shaken (250 rpm) post-induction for 18–22 h at 18 °C in complete darkness, harvested by centrifugation, and dissolved in 50 mL of lysis buffer (50 mM sodium phosphate, 500 mM NaCl, 0.5% Triton X-100, pH 6.5) per liter of harvested culture.

**In vitro refolding.** His6-Gb1-tagged BcLOV4 DUF (Δ1–356) was expressed in BL21(DE3) E. coli. After lysing cells with a French Pressure Cell (Avestin)

Fig. 5. Light-regulated membrane localization in yeast (S. cerevisiae). (A) Spinning-disk confocal fluorescence micrographs of BcLOV4-mCherry in transformed yeast show reversible membrane localization in response to blue light. Cells were immobilized on agar pads immediately before imaging. (Scale bar: 5 μm.) (B) Population analysis of membrane association kinetics. Time constants determined from relative fraction of membrane-bound BcLOV4 from cellular line sections (mean ± SEM; n = 31 cells; τoff = 1.20 s; 95% CI, 1.07–1.38 s). (C) Population analysis of membrane dissociation kinetics (τoff = 84.9 s; 95% CI, 83.3–93 s).
Eukaryotic Cellular Assays.

**Mammalian cell culture and transfection.** HEK293T cells were cultured in D10 media and maintained at 37 °C in 5% CO2 incubator. Cells were seeded onto collagen-treated or poly-o-lysine-treated glass bottom dishes or into 24-well glass-bottom plates, and transfected at 20–30% confluence using the TransIT-293 transfection reagent. Cells were imaged 24–48 h posttransfection.

**Yeast strains were immobilized on agarose pads** (455 nm, −20,300 M−1 cm−1) for 5–10 min, supernatant was added drop-by-drop to 500 mL of dilution buffer (50 mM Tris HCl (pH 8), 500 mM NaCl) over a 2–3-h period to refold the protein. Protein was concentrated via Amicon stirred cell and FPLC-purified on a Superdex 75 or 200 size exclusion column.

**In Vitro Protein Analysis: Nonlipid Interactions.**

**Absorbance spectroscopy and photocycling measurements.** Absorbance scans were measured on a Jasco V-570 spectrophotometer. Fluorescence measurements were performed on an automated Leica DMI6000B fluorescence microscope as described previously (64). When needed, isoprenylated GFP (66) was co-transfected as a plasma membrane marker. After a 5–6-h long blue light pulse (15 mM/cm²), BcLOV-mCherry images were collected every 200 ms (membrane association) or 5 s (membrane dissociation). Localization kinetics was measured for single cells by line section analysis in ImageJ and MATLAB. Spinning-disk confocal microscopy was described previously (65).

**HEK cell fixation and immunocytochemistry.** Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, under dim red light (dark-adapted) or strobed illumination (Mitoxant; λ = 455 nm, 15 mM/cm², 5-s on/25-s off). Immunocytochemistry analysis of 3×FLAG-tagged protein was performed with standard methods with Alexa 488-conjugated anti-3×FLAG (5407; Cell Signaling Technology) or anti-3×FLAG antibody (8146; Cell Signaling Technology) followed by an Alexa 488-conjugated secondary antibody (#4408; Cell Signaling Technology).

**Diffusion estimates.** The intracellular diffusion constant \((D_{intr})\) of BcLOV-mCherry was calculated at 8.8±2 cm²/s, assuming an intracellular diffusion constant of 10 nm measured by DLS and an intracellular viscosity of 2.5 centipoise in mammalian cells (67). The timescale for diffusion to the plasma membrane was considered the time to travel a length of a cell radius \(r_{cell}\) in two dimensions \(\sim r_{cell}/(D_{intr})\) (68), assuming a 5- to 7.5-µm radius for HEK cells. The diffusion timescale in HEK was estimated as 0.7–1.6 s. Diffusion timescales in yeast (estimated as 0.5–1 sol) were calculated similarly assuming a 2- to 3-µm radius and cyttoplasmic viscosity of 10 centipoise (69).

**In Vitro Protein Analysis: Lipid Interactions.**

**Absorbance spectroscopy and photocycling measurements.** Absorbance scans were measured on a Jasco V-570 spectrophotometer. Photocycle kinetics was measured by monitoring the absorbance at 450 nm (A450); after subtracting the absorbance at 565 nm, the intracellular diffusion constant \((D_{intr})\) for 5-s on/25-s off). Immunocytochemistry analysis of 3×FLAG-tagged protein was performed with standard methods with Alexa 488-conjugated anti-3×FLAG (5407; Cell Signaling Technology) or anti-3×FLAG antibody (8146; Cell Signaling Technology) followed by an Alexa 488-conjugated secondary antibody (#4408; Cell Signaling Technology).

**Diffusion estimates.** The intracellular diffusion constant \((D_{intr})\) of BcLOV-mCherry was calculated at 8.8±2 cm²/s, assuming an intracellular diffusion constant of 10 nm measured by DLS and an intracellular viscosity of 2.5 centipoise in mammalian cells (67). The timescale for diffusion to the plasma membrane was considered the time to travel a length of a cell radius \(r_{cell}\) in two dimensions \(\sim r_{cell}/(D_{intr})\) (68), assuming a 5- to 7.5-µm radius for HEK cells. The diffusion timescale in HEK was estimated as 0.7–1.6 s. Diffusion timescales in yeast (estimated as 0.5–1 sol) were calculated similarly assuming a 2- to 3-µm radius and cyttoplasmic viscosity of 10 centipoise (69).

**Apo-protein extinctions.** The apo-protein extinction coefficients were calculated using ExPASy-ProtParam (71). Reported concentration is for holoprotein.

**In Vitro Protein Analysis: Nonlipid Interactions.**

**Absorbance spectroscopy and photocycling measurements.** Absorbance scans were measured on a Jasco V-570 spectrophotometer. Photocycle kinetics was measured by monitoring the absorbance at 450 nm (A450); after 15 s of baseline measurements, samples were stimulated with a collimated LED (Mitoxant; 10 s, λ = 455 nm, 15 mM/cm²), and recovery was monitored in the dark. For solid-phase photocycling measurements, 40 ng of His6-tagged BcLOV4-mCherry proteins in HBS-N were passed over the chip surface (30 µL/min for 10 min). The 100 mM NaOH was injected (50 µL/min for 1 min) to regenerate the SUV-coated chip after each binding experiment. Steady-state equilibrium values were analyzed in MATLAB. Water-in-oil emulsions were formed by vortexing a mixture of 20 mM total lipids (total in decane) with 1.28 µL of purified mCherry-tagged protein in PBS. Twenty microliters of the mixture were transferred to microwells and imaged with a 20× objective. Automated MATLAB scripts were used to segment the interface vs. dispersed phase, and to calculate fluorescence over these regions (area-normalized) and their ratios. All ratios were normalized to illuminated mCherry for illumination measurements.

**Protein–lipid overlay assay.** Blots were created based on methods reported by others (73), using 1 µL of 3 mM phospholipid per spot, and probed with mouse anti-His primary antibody (2366; Cell Signaling Technology) and IRDye 680RD goat (polyclonal) anti-mouse IgG (925–68070; LI-COR). Processed blots were imaged on an Odyssey Infrared Imaging System.

**Bioinformatics.**

**Maximum-likelihood phylogenetic tree construction.** The tree was constructed by aligning all candidate sequences with MUSCLE, building a phylogenetic tree with PhyML, and rendering a tree with TreeDyn through the phylogeny.fr webserver (www.phylogeny.fr) (74). Taxonomic class assignments were made with the Interactive Tree of Life (ITOL) server (itol.embl.de) (75).

**Secondary-structure modeling and consensus annotation.** Candidate amino acid sequences were submitted individually to I-TASSER (76), Jpred (77), Phyre (78), and PSIPRED (79). A consensus secondary-structure prediction was generated by equally weighting a-helix and b-sheet predictions from the four algorithms at every amino acid residue and requiring two of four programs to agree on any given structural element. Amphipathic helices were predicted with the HeliQuest webserver (80).

**De novo energy minimization modeling in Rosetta.** De novo structural predictions were performed with Rosetta, version 3.8, on 100 Intel E5-2665 2.4-GHz Xeon processors using the Ab initio Relax protocol. The consensus secondary-structure prediction was used throughout the process to filter out trajectories that were unlikely to converge to the supplied secondary structure. Near-native topologies were identified by determining the most frequently sampled conformations using clustering with rmsd as the distance metric. The lowest energy trajectory of the largest cluster was hypothesized to be the closest approximation of the native structure.

**ACKNOWLEDGMENTS.** We thank Ivan Kuznetsov for computational structural predictions in Rosetta, the Dan Hammar laboratory for FPLC access, the Matt Good laboratory for technical assistance with emulsion studies, the Youhai Chen laboratory and the Wistar Institute molecular screening facility for technical assistance with liposome preparation and SPR, and Casey Sniffin for technical assistance. We also thank Ranganath Parthasarathy, Julia Schumacher, and Richard Neubig for helpful discussion. B.Y.C. acknowledges the support of National Science Foundation (NSF) Systems and Synthetic Biology (MCB 1652003), NSF Biophotonics (CBET 126497), NIH/National Institute on Drug Abuse (R21 DA040434), Penn Medicine Neuroscience Center, W. W. Smith Charitable Trust for the Heart, NIH/National Institute of Neurological Disorders and Stroke (NINDS) (R01 NS101106), and Defense Advanced Research Projects Agency (Living Foundries S710003185). K.H.G. acknowledges support from NIH/National Institute of General Medical Sciences (R01 GM106239). S.T.G. acknowledges the fellowship support of the NSF Graduate Research Fellowship Program. C.E.B. acknowledges support from the Penn Center for Advanced Therapeutics Training Grant (NIH/NINDS, T32 NS051906). B.S.S. acknowledges support from an NIH postdoctoral fellowship (F32GM119430).