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The Ubiquitin-like Protein PLIC-2 Is a Negative Regulator of G Protein-coupled Receptor Endocytosis

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The activity of many signaling receptors is regulated by their endocytosis via clathrin-coated pits (CCPs). For G protein-coupled receptors (GPCRs), recruitment of the adaptor protein arrestin to activated receptors is thought to be sufficient to drive GPCR clustering in CCPs and subsequent endocytosis. We have identified an unprecedented role for the ubiquitin-like protein PLIC-2 as a negative regulator of GPCR endocytosis. Protein Linking IAP to Cytoskeleton (PLIC)-2 overexpression delayed ligand-induced endocytosis of two GPCRs: the V2 vasopressin receptor and β-2 adrenergic receptor, without affecting endocytosis of the transferrin or epidermal growth factor receptor. The closely related isoform PLIC-1 did not affect receptor endocytosis. PLIC-2 specifically inhibited GPCR concentration in CCPs, without affecting membrane recruitment of arrestin-3 to activated receptors or its cellular levels. Depletion of cellular PLIC-2 accelerated GPCR endocytosis, confirming its regulatory function at endogenous levels. The ubiquitin-like domain of PLIC-2, a ligand for ubiquitin-interacting motifs (UIMs), was required for endocytic inhibition. Interestingly, the UIM-containing endocytic adaptors epidermal growth factor receptor protein substrate 15 and Epsin exhibited preferential binding to PLIC-2 over PLIC-1. This differential interaction may underlie PLIC-2 specific effect on GPCR endocytosis. Identification of a negative regulator of GPCR clustering reveals a new function of ubiquitin-like proteins and highlights a cellular requirement for exquisite regulation of receptor dynamics.

INTRODUCTION

Regulation of G protein-coupled receptor (GPCR) surface levels through endocytosis plays a critical role in the cellular sensitivity to extracellular stimuli. By removing activated surface GPCRs, regulated endocytosis contributes to rapid signal termination (or desensitization), initiates new signaling pathways, and/or enables receptors to recover their signaling abilities (Sorkin and Von Zastrow, 2002; von Zastrow, 2003; Wolfe and Trejo, 2007). Ligand-induced endocytosis of GPCRs typically proceeds in several distinct steps. First, activated GPCRs are rapidly phosphorylated and recruit cytoplasmic proteins called arrestins. Second, receptor–arrestin complexes are concentrated in clathrin-coated pits (CCPs). Finally, receptors are internalized when the CCPs undergo dynamin-dependent endocytic scission.

The traditional view of GPCR endocytosis is that it is regulated mainly at the level of recruitment of arrestin. Arrestins 2 and 3 (often called β-arrestins or nonvisual arrestins) bind directly both to phosphorylated GPCRs and to components of the clathrin machinery such as clathrin and adaptor protein (AP)2, thereby functioning as endocytic adaptors that localize GPCRs to clathrin-coated pits (Reiter and Lefkowitz, 2006; DeWire *et al*., 2007; Premont and Gainetdinov, 2007; Traub and Lukacs, 2007). Endocytosis of most proteins, once they localize to CCPs, is thought to occur "by default." Therefore, phosphorylation-dependent binding of arrestin to GPCRs is thought to be sufficient to drive the series of events that culminate in receptor internalization.

Recent data, however, suggest that regulated endocytosis is more complex. On activation, GPCRs localize only to a specified subset of coated pits. Furthermore, some GPCRs can modulate the lifetimes of CCPs even after clustering; as a result, they regulate their own endocytosis (Lakadamyali *et al*., 2006; Mundell *et al*., 2006; Puthenveedu and von Zastrow, 2006). Therefore, an alternate hypothesis is that distinct mechanisms actively regulate each step of the process to fine-tune the kinetics of endocytosis. The present work provides evidence that GPCR endocytosis is regulated downstream of arrestin recruitment to activated receptors.

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Abbreviations used: β2AR, β2-adrenergic receptor; CCP, clathrin-coated pit; EGFR, epidermal growth factor receptor; Eps15, epidermal growth factor receptor protein substrate 15; PLIC, protein linking IAP to cytoskeleton; TfR, transferrin receptor; UBA, ubiquitin-associated-; UbL, ubiquitin-like; UIM, ubiquitin-interacting motif; V2R, V2 vasopressin receptor.

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We have identified Protein Linking IAP (CD47) to cytoskeleton (PLIC-2), also called ubiquilin-2, as a negative regulator of GPCR/arrestin clustering in CCPs. Inhibited clustering of GPCR–arrestin complexes in CCPs, mediated by the ubiquitin-like (UbL) domain of PLIC-2, leads to delaying endocytosis of GPCRs specifically, but not of other membrane cargo tested. Our results identify a novel function of a ubiquitin-like protein in inhibiting, rather than promoting, endocytosis of GPCRs. They further suggest that multiple mechanisms, in addition to recruitment of arrestins by activated GPCRs, function in fine-tuning the regulated endocytosis of this large and important family of signaling receptors.

MATERIALS AND METHODS

Antibodies and Reagents

Transferrin receptor antibody (clone OKT9) was from eBiosciences (San Diego, CA). Alexa 647-coupled epidermal growth factor (EGF), Alexa-coupled secondary antibodies, and Prolong mounting medium were from Invitrogen (Carlsbad, CA). The arrestin antibody was from Affinity BioReagents (Golden, CO). FLAG antibodies (M1) phycoerythrin (PE)-coupled anti-mouse antibody, and isoproterenol were from Sigma-Aldrich (St. Louis, MO). Myc tag antibody (clone 9E10) was from Upstate Biotechnology (Charlottesville, VA). Arginine vasopressin (AVP) was from Bachem California (Torrance, CA). The green fluorescent protein (GFP), epidermal growth factor receptor protein substrate 15 (Eps15), and Epsin (1 and 2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PLIC antibodies were characterized previously (Wu *et al*., 1999).

DNA Constructs

The Flag-tagged human V2 receptor (V2R) and Flag-tagged human β_2-adrenergic receptor expressed in pcDNA3.1 have been described previously (Cao *et al*., 1999; Klein *et al*., 2001). Eps15Δ95/295 was previously characterized as a dominant-negative construct (Benmerah *et al*., 1999). The GFP-arrestin3 cDNA was from Marc Caron (Duke University, Durham, NC). GFP-Dynamin K44AE was provided by D. Fortin (University of California, San Francisco). The DsRed-clathrin cDNA was described previously (Merrifield *et al*., 2002; Puthenveedu and von Zastrow, 2006).

PLIC Fusion Proteins

The GST-PLIC constructs have been described previously (Wu *et al*., 1999). To generate GFP-tagged PLIC constructs, PLIC-1 and PLIC-2 coding sequences from GST-PLIC constructs (Wu *et al*., 1999) were excised using EcoRI/SalI for PLIC-1 or SacI/Hind III for PLIC-2, and they were ligated into the pEGFP-C3 vector (BD Biosciences, San Jose, CA).

PLIC-2ΔUbL (amino acids [aa] 104-634) was generated by polymerase chain reaction (PCR) by using the following primers: 5′-GCACGCCGAATTCTTAGCCAGAACCGTCCGCAG-3′ and 5′-GCCAGACCTCGAGTTAGGATGGCTGAGAGCCCAGCAG-3′. To generate GFP-PLIC-2 ΔUBA (aa 1-579), the following PCR primers were used: 5′-CCGCCTTGAATTCTTATGGCTGAGAACGGCGAGA-3′ and 5′-CCGCCTCGTCGACCTATTACACTTCAGGATTCGGCGGC-3′.

The PCR fragments were digested with EcoRI/XhoI (PLIC-2 ΔUbL) or EcoRI/SalI (PLIC-2 ΔUBA), and they were ligated into the vector pEGFP-C3 linearized with EcoRI/SalI. All constructs were sequenced to verify the absence of errors.

Cell Culture and Transfection

HeLa and human embryonic kidney (HEK)-293 cells were maintained in DME-H21 medium supplemented with 10% fetal calf serum. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Experiments were performed 24 h post-transfection. 293 cells stably expressing the β2-adrenergic receptor (β2AR) were maintained under selection in 500 μg/ml G-418 (Geneticin; Invitrogen) and were characterized previously (Gage *et al*., 2005).

GPCR Internalization Assay

Quantification of receptor internalization was obtained by measuring cell surface receptor expression with and without agonist treatment by flow cytometry, as described previously (Hanyaloglu *et al*., 2005; Hanyaloglu and von Zastrow, 2007). Briefly, transfected HeLa cells were incubated with M1 anti-FLAG antibody followed by agonist treatment for 0–45 min, then they were detached with trypsin, incubated with a PE-coupled anti-mouse secondary antibody (Sigma-Aldrich), and analyzed by flow cytometry (FACSCalibur; BD Biosciences). For each sample, 10,000 cells were counted, and the PE fluorescence intensity of GFP-positive cells was measured. All time points were carried out in duplicates, and the experiment was performed at least three times. The percentage of internalized receptor was determined by subtracting the percentage of cell surface levels of receptor after addition of agonist compared with levels in untreated cells.

For PLIC knockdown experiments, HeLa cells plated in 10-cm culture dishes at 40% confluence were transfected with 50 nM nonsilencing small interfering RNA (siRNA) (target sequence AATTCTCCGAACGTGTCACGT; QIAGEN, Valencia, CA), siRNA for PLIC-1 (targeted sequence GAAGAAATCTCTAAACGTTTT; Dharmacon RNA Technologies, Lafayette, CO), or siRNA for PLIC-2 (targeted sequence TCCCATAAAGAGACCCTAATA; QIAGEN) by using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, cells were cotransfected with 50 nM siRNA and 12 μg of V2R cDNA. Cells were replated in 12-well plates, and they were used the next day for endocytosis assays as described above. Depletion was confirmed by Western blot by using anti PLIC-1 or PLIC-2 antibodies.

GFP-Arrestin3 Recruitment Assay

HeLa cells transfected with Flag-V2R, GFP-arrestin3, and, where indicated mycPLIC-2, were plated onto coverslips 4–6 h after transfection. After incubation with a rabbit Flag antibody (3.5 μg/ml; 20 min; 37°C), agonist treatments (10 μM AVP) were carried out for 0, 1, or 5 min. Cells were fixed, permeabilized, and stained for V2R and mycPLIC-2, and observed under a confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) with a 63× oil objective, numerical aperture of 1.4. Twenty cells per condition were imaged, and arrestin localization was categorized as described in text.

Glutathione Transferase (GST) Pull-Down Assays

The assays were performed essentially as described previously (N'Diaye and Brown, 2003). Briefly, bead-bound GST-PLIC-1 or PLIC-2 was incubated with HeLa cell lysates overnight at 4°C under constant agitation. GST, alone or in fusion with syntaxin-2, was used as negative control. The bead-bound material was analyzed by Western blot with antibodies against Eps15, Epsin-1, or Epsin-2.

EGF Internalization

HeLa cells transfected with the indicated GFP-tagged proteins: PLIC-1, PLIC-2, or Dynamin K44E) were serum-starved for 1 h at 37°C in 0.1% bovine serum albumin-containing DME. Alexa 647-EGF (100 ng/ml) was added for 10 min. At endpoint, cells were washed, and bound EGF was stripped off the membrane by addition of 50 mM acetic acid and 150 mM NaCl, pH 2.9. To control for residual membrane-bound EGF, a set of cells were incubated at 4°C with labeled EGF, and then they were incubated or not in acid strip buffer. After a phosphate-buffered saline (PBS) wash, cells from each assay condition were lifted off the plate with trypsin, fixed in 2% paraformaldehyde, and the EGF fluorescence associated with the GFP-positive population was analyzed by flow cytometry (FACSCalibur; BD Biosciences). The fluorescence of membrane-bound EGF was subtracted from all samples.

Transferrin Receptor Analysis

To analyze PLIC effect on steady-state levels of transferrin receptor, HeLa cells transfected with the indicated GFP-tagged proteins: PLIC-1, PLIC-2, or dominant-negative Eps15 was lifted off and incubated with a transferrin receptor antibody (clone OKT-9; 5 μg/ml) for 1 h at 4°C. Cells were rinsed twice with PBS and incubated with a PE-coupled anti-mouse immunoglobulin G secondary antibody for 30 min at 4°C. Cells were rinsed, and the PE fluorescence on the transfected cell population measured by flow cytometry (FACSCalibur; BD Biosciences).

Total Internal Reflection Fluorescence (TIRF) Microscopy Live Imaging

Imaging was carried out using a Nikon TE-2000E inverted microscope with a 60× TIRF objective (numerical aperture 1.45), and equipped for through-the-objective TIRF illumination. A 488-nm argon-ion laser (Melles Griot, Carlsbad, CA) and a 543-nm HeNe laser (Spectra Physics, San Jose, CA) were used as light sources. Transfected cells were imaged in Opti-MEM (Invitrogen) with 2% serum and 30 mM HEPES, pH 7.4, maintained at 37°C using a temperature-controlled stage (Bioscience Tools, San Diego, CA) and an objective warmer (Bioptechs, Butler, PA). Time-lapse sequences were acquired using a C9100-12 camera (Hamamatsu Photonics, Bridgewater, NJ) driven by IPLab (Scanalytics, Fairfax, VA). To quantify receptor clustering in PLIC-2–transfected cells, samples were fed with M1 anti-Flag antibody (20 min; 37°C) before agonist stimulation for 1 or 4 min. Cells were then washed in ice-cold PBS and fixed (−20°C methanol; 5 min).

Statistical Analysis

Each experiment was repeated at least three times. Statistical significance was determined using paired Student's *t* test; differences were considered significant at $p \leq 0.05$.

RESULTS

Overexpression of PLIC-2 Inhibits GPCR Endocytosis

A role for PLIC-2 in regulated endocytosis was first observed by monitoring the trafficking of the V2R, a GPCR whose internalization is known to occur via clathrin-coated pits (Pfeiffer *et al*., 1998; Oakley *et al*., 1999; Klein *et al*., 2001). Among the PLIC family, PLIC-1 and PLIC-2 are both ubiquitously expressed and predominantly cytosolic in their localization (Wu *et al*., 1999). HeLa cells were cotransfected with Flag-tagged V2R and either GFP, GFP-PLIC-1, or GFP-PLIC-2. Fluorescence flow cytometry has been used previously to accurately quantify changes in surface receptor fluorescence in response to agonist in a large population of cells (10,000) (Tanowitz and von Zastrow, 2003; Gage *et al*., 2005; Hanyaloglu *et al*., 2005; Hanyaloglu and von Zastrow, 2007), and it allows the specific advantage of analyzing only those cells coexpressing Flag-tagged receptors and GFP-tagged proteins. In GFP and Flag-V2R–expressing cells, the addition of agonist (0–45 min) resulted in a loss of surface receptor, with a $t_{1/2}$ of $\sim$ 7 min, similar to rates previously shown for the V2R (Pfeiffer *et al*., 1998), and it was not significantly different from cells expressing only Flag-V2R (data not shown). However, coexpression of GFP-PLIC-2 resulted in a dramatic delay in the rate of V2R internalization ($t_{1/2} \sim 30$ min; Figure 1A). This effect of PLIC-2 was specific, because expression of PLIC-1 at similar levels had no effect on V2R endocytosis (Figure 1A). We also analyzed the role of PLIC-2 for another GPCR that undergoes clathrin-mediated internalization, the β2AR (Moore *et al*., 1995; Goodman *et al*., 1996; Cao *et al*., 1999; Laporte *et al*., 1999; Puthenveedu and von Zastrow, 2006). Like the V2R, ligand-dependent internalization of the Flag-β2AR was significantly delayed in cells coexpressing PLIC-2 but not PLIC-1 (Figure 1B; $t_{1/2} \sim 6$ min in control to $\sim$15 min in PLIC-2 cells).

To determine the generality of the effect of PLIC-2 expression on receptor endocytosis, we measured internalization of additional membrane cargo, the transferrin receptor (TfR), and EGF receptor (EGFR). TfR, a well-known marker for clathrin-mediated endocytosis, is constitutively internalized and recycled. Therefore, we measured steady-state levels of surface receptor in cells expressing GFP-tagged PLIC-1 or PLIC-2 as an indicator of TfR endocytosis, because an inhibition in endocytosis will result in increased surface levels of TfR (Benmerah *et al*., 1999). Interestingly, PLIC-1 or PLIC-2 overexpression had no effect on steady-state surface levels of TfR (Figure 1C). A truncated mutant of Eps15 (DN Eps15), lacking the second and third Eps15 homology domains, has been shown previously to produce a dominant-negative inhibition of TfR endocytosis via clathrin-coated pits by sequestering the clathrin adaptor AP2 (Benmerah *et al*., 1999). Expression of DNEps15 is significantly inhibited TfR endocytosis as indicated by the increased surface levels of TfR at the plasma membrane (Benmerah *et al*., 1999; Figure 1C). Internalization of the EGFR was assessed by quantifying the uptake of fluorescently labeled EGF by flow cytometry in cells expressing GFP-tagged PLICs. Because EGFR is reported to internalize either through CCPs or caveolae (Sigismund *et al*., 2005), we expressed a dominant-negative dynamin K44E (DynK44E) mutant, which inhibits

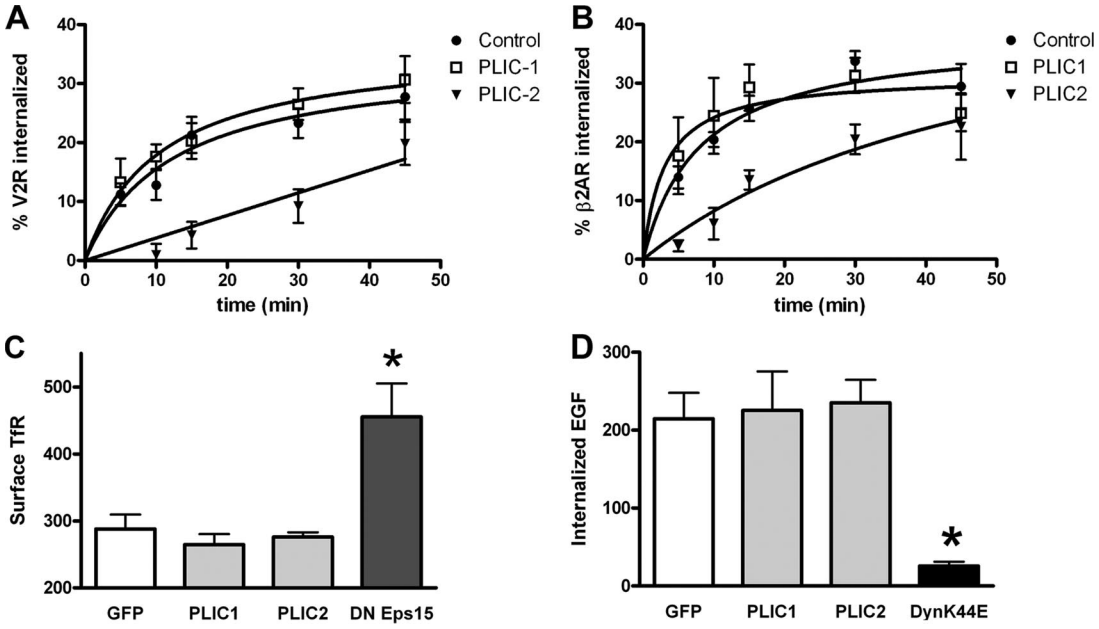


Figure 1. Overexpression of PLIC-2, but not PLIC-1, inhibits GPCR internalization. (A) HeLa cells were transfected with FLAG-V2R and GFP (control), GFP-PLIC-1, or GFP-PLIC-2. To label surface receptors, cells were fed with M1 anti-FLAG antibody before stimulation with AVP for the indicated times. Receptor internalization in cotransfected cells in each of these groups was quantified by flow cytometry. All experiments were performed in duplicate. Graph represents a mean ± SE of seven independent experiments. (B) HeLa cells coexpressing FLAG-β2AR with GFP, GFP-PLIC-1, or GFP-PLIC-2 were fed with M1 anti-FLAG antibody before stimulation with isoproterenol for the indicated times. Receptor internalization was measured as described in A. Graph represents the mean ± SE of 3 independent experiments. (C) Steady-state levels of TfR were measured by flow cytometry in HeLa cells transfected with GFP, GFP-PLIC-1, GFP-PLIC-2, or GFP-tagged DN Eps15. This graph represents a mean ± SE of four independent experiments, with *$p < 0.05$. (D) HeLa cells transfected with GFP, GFP-PLIC-1, GFP-PLIC-2, or GFP-tagged dynamin K44E were stimulated with Alexa 647-EGF for 10 min. After removal of membrane-bound EGF, ligand internalization was quantified by flow cytometry. Graph represents a mean ± SE of four independent experiments, with *$p < 0.05$.

both modes of uptake, as a control for inhibition of endocytosis (Herskovits *et al.*, 1993). Expression of DynK44E strongly inhibited EGF internalization, whereas expression of either PLIC isoform did not (Figure 1D). Thus, the ability of PLIC-2 overexpression to significantly delay endocytosis of the V2R and β2AR but not other membrane cargo (TfR and EGFR) strongly suggests that PLIC-2 functions selectively to regulate clathrin-mediated endocytosis of GPCRs.

The UbL Domain of PLIC-2 Is Required for Regulation of GPCR Endocytosis

Both PLIC-1 and PLIC-2 are multidomain proteins containing an amino-terminal UbL domain and a carboxy-terminal ubiquitin-associated (UBA) domain (Figure 2A). The UBA domain interacts with ubiquitinated chains (Bertolaet *et al.*, 2001; Wilkinson *et al.*, 2001), whereas the UbL domain functions as a ligand for ubiquitin-interacting motifs or UIMs present in proteins associated with the proteasome (Walters *et al.*, 2002) and endocytic adaptors (Regan-Klapisz *et al.*, 2005). Given the role of protein ubiquitination in promoting GPCR endocytosis (Shenoy *et al.*, 2001; Hicke and Dunn, 2003) and to gain mechanistic insight into the action of PLIC-2, we next determined which of these regions of the protein conferred the inhibitory effect on GPCR endocytosis. GFP-tagged deletion mutants of PLIC-2 were generated (Figure 2A), and they were tested for their ability to inhibit V2R internalization when overexpressed. These mutant constructs were expressed at similar levels to full-length GFP-PLIC-2 (as determined by flow cytometry; data not shown). In addition to being soluble in the cytosol, full-length PLIC-2 is mainly found in cytoplasmic aggregates, similar to those observed for PLIC-1 (Regan-Klapisz *et al.*, 2005). Truncation of the N-terminal UbL domain produced a mutant PLIC-2 with similar cellular localization to full-length PLIC-2 (Figure 2B). However, this mutant was defective in its ability to inhibit V2R internalization after agonist treatment (Figure 2C). In contrast, deletion of the UBA domain produced a mutant PLIC-2 that was still capable of inhibiting V2R internalization to similar levels as full-length PLIC-2 (Figure 2C), even though its localization was mainly cytosolic (Figure 2B), suggesting that the inhibitory effect on V2R endocytosis is not dependent on PLIC-2 localization to cytoplasmic aggregates.

Overall, these data indicate that the UbL, but not the UBA domain of PLIC-2, is required for its inhibitory effect on GPCR endocytosis, and they suggest that a UIM-containing endocytic protein might be involved in PLIC-2 regulation of GPCR endocytosis.

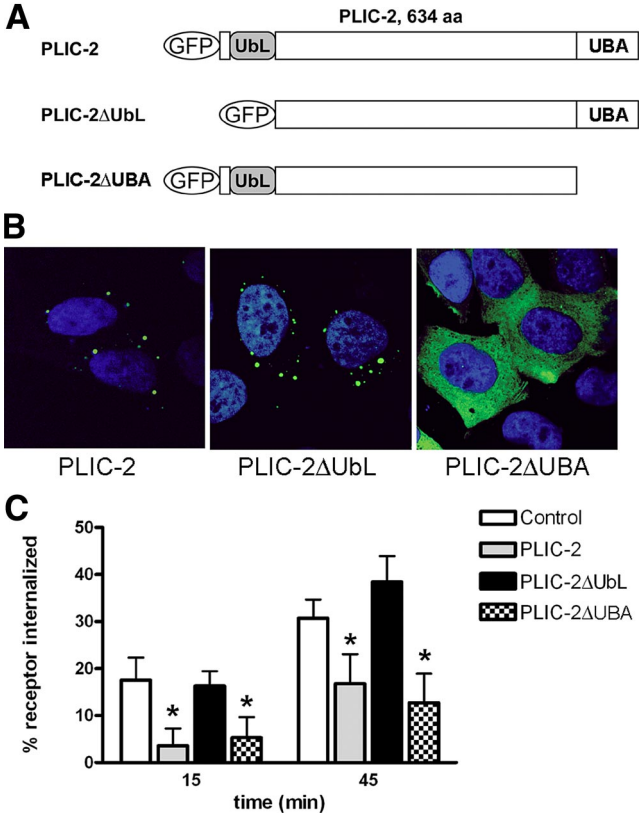


Figure 2. The UbL domain of PLIC-2 is required for its effect on V2R endocytosis. (A) Schematic of PLIC-2 domain deletion mutagenesis. Full-length PLIC-2, PLIC-2ΔUbL, and PLIC-2ΔUBA deletion mutants were generated as GFP-tagged N-terminal fusion proteins. (B) Each GFP-tagged PLIC-2 mutant was expressed in HeLa cells, and its localization was analyzed by confocal microscopy. Representative images are shown from >40 cells analyzed per transfection across six independent experiments. 4,6-Diamidino-2-phenylindole staining of the nucleus is shown in blue. (C) HeLa cells were cotransfected with FLAG-V2R and either GFP (control), GFP-PLIC-2, GFP-PLIC-2ΔUBA or GFP-PLIC-2ΔUbL. Receptor internalization was quantified 15 and 45 min after AVP addition as described in 1. All experiments were performed in duplicate, and the graph represents the mean ± SE of six independent experiments. *$p < 0.05$, statistically different from controls.

PLIC-2 Interacts with the UIM-containing Endocytic Adaptors Eps15 and Epsin

The results mentioned above indicate that expression of PLIC-2 but not PLIC-1 inhibits GPCR endocytosis (Figure 1) and that this inhibition by PLIC-2 is dependent on the UbL domain (Figure 2). Because the UbL domain, present in both PLIC-1 and PLIC-2, is a known ligand for UIMs (Walters *et al.*, 2002; Regan-Klapisz *et al.*, 2005), we sought to identify possible UIM-containing proteins that would interact specifically with PLIC-2. Pull-down assays were performed in which GST-PLIC-1 or GST-PLIC-2 was incubated with HeLa cell lysates. Both PLIC-1 and PLIC-2 interacted with Eps15 in vitro (Figure 3A). However, Eps15 showed increased binding with PLIC-2 compared with PLIC-1 (Figure 3B), suggesting that PLIC-2 may bind Eps15 with a higher affinity than PLIC-1. In contrast to Eps15, Epsin (1 and 2) exhibited a specific interaction with PLIC-2, because no binding was detectable with PLIC-1 under similar experimental conditions (Figure 3A). Thus, these data identify preferential interaction of Epsin and Eps15 with PLIC-2 over PLIC-1. These UIM-containing proteins may represent possible endocytic adaptors involved in PLIC-2 regulation of receptor endocytosis.

PLIC-2 Overexpression Does Not Prevent Arrestin Translocation to the Plasma Membrane but Delays GPCR/Arrestin Coclustering into CCPs

The classical model for regulated endocytosis of GPCRs involves a central role for nonvisual arrestins in receptor recruitment to CCPs (Wolfe and Trejo, 2007; Premont and Gainetdinov, 2007). By their ability to bind both to AP2 and clathrin, these arrestins mediate the association of activated receptors to CCPs before their internalization (Goodman *et al.*, 1996; Laporte *et al.*, 2000). There are several steps during clathrin-mediated endocytosis, from receptor activation to scission of receptor-containing CCPs that PLIC-2 could be inhibiting. To determine which step in endocytosis was affected by PLIC-2 expression, we

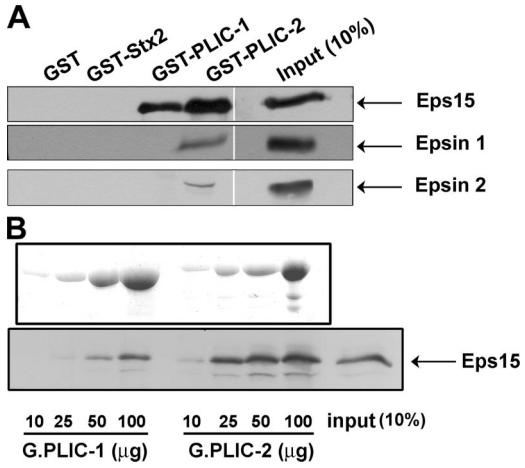


Figure 3. PLIC-2 interacts with Eps15 and Epsin. (A) Comparable amounts of bead-bound PLIC-1 or PLIC-2 in fusion with GST were incubated with HeLa cell lysates. GST alone or in fusion with Syntaxin-2 (Stx2) was used as negative controls. Binding of each construct to Eps15 or Epsin was analyzed by Western blot with antibodies specific to Eps15, Epsin 1, or Epsin 2. (B) Ten to 100 μg of bead-bound GST-PLIC-1 or GST-PLIC-2 (top gel, Coomassie Blue staining) were incubated with a constant amount of HEK-293 cell lysate. Bead-bound material was loaded on a SDS-polyacrylamide gel electrophoresis gel and assessed for PLIC-associated Eps15 by Western blot (bottom gel).

monitored the agonist-dependent trafficking of GFP-tagged arrestin-3 (βARR-2) from its initial translocation to the plasma membrane (Figure 4A, cytosol to membrane smooth), its subsequent plasma membrane clustering (membrane cluster), and coendocytosis with Flag-V2R (Figure 4A, internal cluster).

Arrestin translocation from the cytosol to the plasma membrane (membrane smooth) was detected as early as 1 min after ligand addition, and it was not significantly different between control and PLIC-2–expressing cells (Figure 4B). After translocation, arrestin clusters on the plasma membrane by localizing to CCPs (Oakley *et al*., 1999; Laporte *et al*., 2000). Clustering of translocated arrestin, which occurred within a minute in control cells, was significantly slower in PLIC-2–transfected cells (Figure 4B). Accordingly, receptor/arrestin internalization was delayed, and the appearance of internal clusters at 5 min was significantly higher in control cells than PLIC-2–transfected cells (Figure 4B), confirming the delay in endocytosis previously observed by flow cytometry in Figure 1. This suggests that the delay is not at the level of arrestin recruitment to the plasma membrane, but at the subsequent clustering of arrestin, and consequently receptor, into clathrin-coated pits.

PLIC-2 Delays Receptor Clustering at the Plasma Membrane

To determine directly whether PLIC-2 delays agonist-induced concentration of receptors in CCPs, TIRF microscopy was used to rapidly monitor GPCR clustering at the plasma membrane in live cells (Steyer and Almers, 2001; Puthenveedu and von Zastrow, 2006). In HEK-293 cells stably expressing Flag-β2AR used previously in TIRF studies (Puthenveedu and von Zastrow, 2006), receptor clustering was evident within 30 s after agonist addition in untransfected cells. In cells expressing GFP-PLIC-2, however, there was a dramatic delay in receptor cluster formation (Figure 5B and Supplemental Video S1). Clustering of Flag-β2AR in these cells was not visible until ~210 s after agonist addition, whereas much more rapid clustering of the GPCR was observed in surrounding (untransfected) cells not expressing GFP-PLIC-2 (Figure 5B and Supplemental Video S1). This effect was specific to PLIC-2, because wild-type GFP (not fused to PLIC-2) did not detectably delay receptor clustering compared with surrounding untransfected cells (Figure 5A and Supplemental Video S2).

To quantify this effect in randomly selected cells from a large population, control (GFP) and PLIC-2–expressing cells were exposed to a saturating concentration of the GPCR agonist for the indicated time period, and then they were fixed and scored for visible receptor clusters by TIRF imaging (Figure 5C). Following 1 min of agonist stimulation, the percentage of cells displaying clustered receptor was significantly lower in PLIC-2–expressing cells compared with GFP control (Figure 5C). After 4 min of agonist stimulation, the inhibitory effect of PLIC-2 on receptor clustering was still significant, but reduced, compared with the earlier time point.

The localization of PLIC-2 in relation to CCPs was also examined by live TIRF microscopy (Supplemental Videos S3 and S4). β2AR-expressing HEK-293 cells were cotransfected with DsRed-clathrin (previously characterized as a marker for CCPs; Merrifield *et al*., 2002; Puthenveedu and von Zastrow, 2006) and GFP-PLIC-2. Colocalization of GFP-PLIC-2 (Supplemental Video S3, top) with DsRed-clathrin (Supplemental S3, bottom) was minimal in untreated cells. Agonist stimulation induced a small increase in the colocalization between PLIC-2 and CCPs (Supplemental Video S4). This observation further supports a role for PLIC-2 in regulating association of activated GPCRs with CCPs.

Because PLIC-2 expression specifically delayed GPCR endocytosis at the level of receptor clustering in to CCPs without affecting arrestin recruitment, we next determined whether PLIC-2 overexpression reduced cellular levels of arrestin that could underlie the observed endocytic effects. The levels of endogenous arrestin were measured by Western blotting from cell lysates transfected with GFP-PLIC-2 (Figure 5D). Overexpression of PLIC-2 had no affect on the levels of arrestin in these cells. Together, these results provide strong evidence that PLIC-2 inhibits GPCR endocytosis by delaying receptor coclustering with arrestin into CCPs.

PLIC-2 Is Required for Negative Regulation of GPCR Endocytosis

We hypothesized that the inhibitory effect of PLIC-2 overexpression on GPCR endocytosis might indicate an essential role for endogenous PLIC-2 in this process, either by inhibiting or promoting GPCR endocytosis. Therefore, we examined the effects of depleting cellular PLIC-2 on GPCR endocytosis by using siRNA. Cellular levels of PLIC-2 and PLIC-1 were substantially reduced by transfection of siRNA specific for each isoform, but not with nonsilencing (control) siRNA (Figure 6A). Knockdown of PLIC-2 in cells expressing Flag-V2R significantly increased the rate of V2R internalization after agonist stimulation (Figure 6B). In contrast, depletion of endogenous PLIC-1 to at least the same extent did not affect the kinetics of receptor internalization (Figure 6B). To gain further evidence for the endogenous role of PLIC-2, and to establish that PLIC-2 exerted its effect on more than one GPCR, depletion studies were extended to the β2AR. Lowering cellular amounts of PLIC-2 in HeLa cells expressing the Flag-β2AR increased the rate of receptor internaliza-

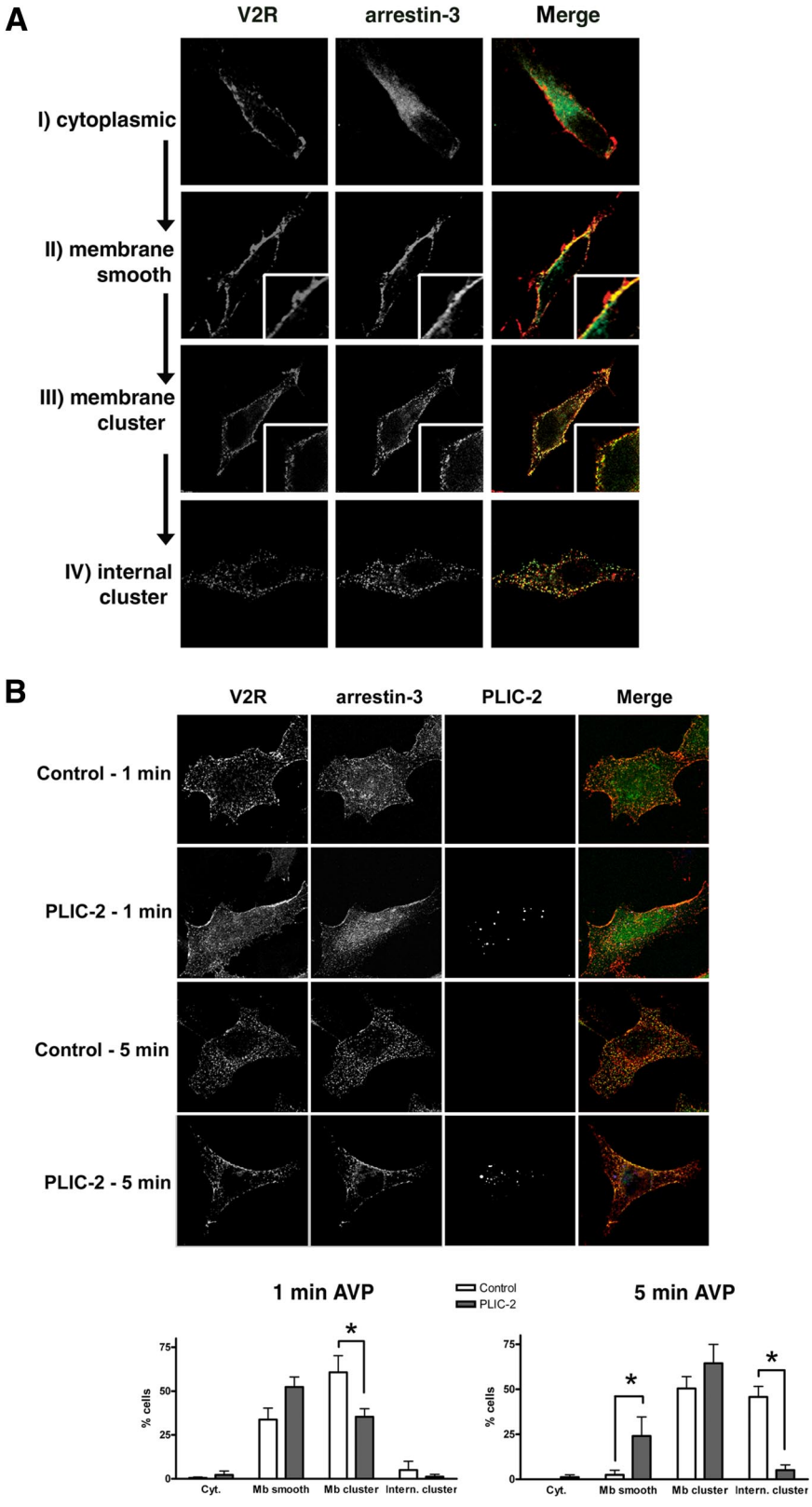


Figure 4. PLIC-2 overexpression does not prevent membrane recruitment of arrestin, but it specifically delays surface clustering of receptor–arrestin complexes. HeLa cells transfected with GFP-arrestin3, FLAG-V2R, and myc-PLIC-2 were stimulated with AVP for 1 or 5 min and compared with control conditions (no PLIC-2). GFP-arrestin3 dynamics upon receptor stimulation were imaged by confocal microscopy and categorized postimaging as indicated in A: cytosolic arrestin (resting state) (I); arrestin translocated to the plasma membrane, uniformly distributed (II); arrestin clustered with V2R into clathrin pits at the plasma membrane (III); and arrestin cointernalized with V2R (IV). Clusters found in cells where sections were taken through the middle of the cell were considered internal. Shown are representative images of cells in each state. (B) Representative images of control (untransfected), and PLIC-2-transfected cells are shown for each stimulation time point. For quantitation analysis, the percentage of cells showing the indicated phenotypes outlined in A after 1 and 5 min of agonist (AVP) stimulation was determined. Graph represents the summary ± SE of four independent experiments, with statistically significant differences (*$p < 0.05$).

tion after agonist stimulation (Figure 6C). As observed with overexpression, cellular levels of arrestin were unaffected by siRNA-mediated depletion of PLIC-2 (Figure 6D). Therefore, although PLIC-2 overexpression delayed receptor endocytosis, depletion of PLIC-2 enhanced the rate of receptor endocytosis. Together, these observations indicate that endogenous PLIC-2 acts as negative regulator of GPCR endocytosis.

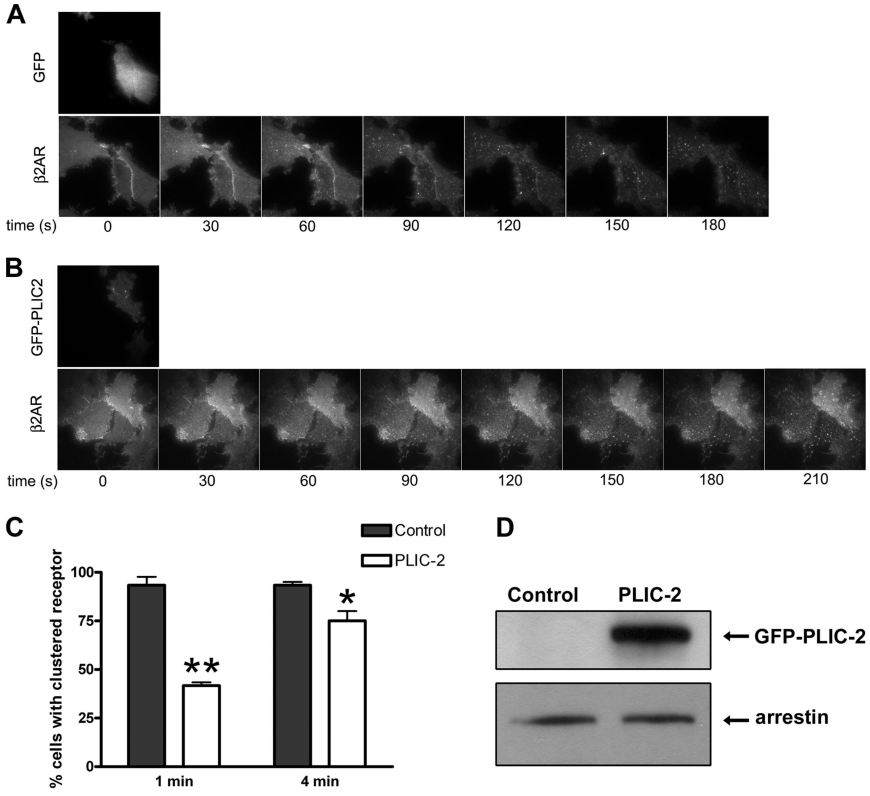


Figure 5. PLIC-2 overexpression delays formation of activated receptor clusters at the plasma membrane. HEK-293 cells stably expressing FLAG-β2AR were transfected with GFP (A) or GFP-PLIC-2 (B), and live imaging was performed using TIRF microscopy. FLAG-β2AR–expressing cells were fed with M1 anti-FLAG conjugated to Alexa Fluor-555 before agonist stimulation (isoproterenol). After stimulation (time 0), formation of membrane clusters by activated receptors was monitored overtime. Images represent still pictures of each sequence (see Supplemental Material) at the indicated time point. Cluster formation was delayed in PLIC-2–expressing cells compared to controls. Quantitation of this delay (C) was achieved by fixing cells after 1 and 4 min of agonist stimulation and scoring cells containing clusters of activated receptors. Graph represents the mean ± SE of three independent experiments, with $^{*}p < 0.05$ and $^{**}p < 0.01$. (D) Cell lysates from HeLa cells transfected with GFP-PLIC-2 (overexpression) were analyzed by Western blot with a GFP (top) or an arrestin (bottom) antibody. Cellular levels of arrestin were compared with those from control (untransfected) cells. Shown is a representative experiment.

DISCUSSION

The present work identifies an unanticipated role for PLIC-2 in modulating the endocytosis of two well-characterized GPCRs, the V2R and β2AR. Regulated endocytosis of these GPCRs is dependent on β-arrestins (arrestins 2 and 3), which function as endocytic adaptors thought to be sufficient in promoting receptor concentration in CCPs. To our knowledge, the present results are the first to identify a cellular protein, PLIC-2, that functions as a negative regulator of endocytosis for GPCRs, by specifically reducing the rate at which GPCR-arrestin complexes concentrate in CCPs. Furthermore, this could provide fundamental insight to the mechanism controlling GPCR endocytosis by demonstrating a requirement for active regulation of receptor concentration in CCPs.

The PLICs are a family of four homologous proteins, each of which contains an amino-terminal UbL domain and a carboxy-terminal UBA domain. PLIC-1 (ubiquilin-1) and PLIC-2 (ubiquilin-2) are predominantly cytosolic proteins (Wu *et al*., 1999), whereas ubiquilin 4 (also called A1U, or ubin) is primarily nuclear (Davidson *et al*., 2000). Ubiquilin 3 is expressed exclusively in testis (Conklin *et al*., 2000) and its subcellular localization has not been examined. Although PLIC proteins were described originally as cytosolic partners for the integral membrane protein CD47 (also called IAP) (Wu *et al*., 1999), subsequent evidence suggested that PLIC proteins function in targeting ubiquitinated proteins to the proteasome (Mah *et al*., 2000; Kleijnen *et al*., 2000; Funakoshi *et al*., 2002). Besides their roles in proteasome targeting, PLICs have been found associated with membranes and components of the cytoskeleton (Wu *et al*., 1999), and it was suggested that they might have other cellular functions in addition to regulating protein degradation (Wu *et al*., 1999). In line with this, we have previously shown that PLIC-1, but not PLIC-2, regulates signaling of GPCRs that are specifically coupled to G_i (N'Diaye and Brown, 2003). These results, together with the present study, suggest that both PLICs have distinct roles in regulating GPCR activity.

Our present results show that PLIC-2 functions as a negative regulator of endocytosis for both the V2R and the β2AR. PLIC-2 overexpression inhibited the rate of agonist-induced internalization of these receptors. Conversely, depletion of endogenous PLIC-2 by using siRNA accelerated agonist-induced endocytosis of these GPCRs. Because PLIC-2 has multiple interaction domains (Wu *et al*., 1999), the overexpression and knockdown results suggest that PLIC-2 levels determine a balance between promoting and inhibiting the rate of agonist-induced internalization, possibly by sequestering associated proteins involved in promoting receptor entry into CCPs. This idea is also consistent with our findings that the UbL domain of PLIC-2 is required for its inhibitory effects on endocytosis. The UbL domain of PLIC-1 has been reported to interact with UIM-containing endocytic adaptors such as Eps15, Eps15R, and hepatocyte growth factor receptor substrate, although functional evidence of a role for PLIC-1 UbL domain in receptor trafficking was not addressed (Regan-Klapisz *et al*., 2005). We have also observed that PLIC-2 binds to similar UIM-containing proteins (Figure 3; data not shown). Given the high homology of the UbL domains between PLIC-1 and -2 it is not unexpected that both ubiquilins would bind similar UIM–containing proteins. However, our data excludes a role for PLIC-1 isoform in regulating GPCR endocytosis, suggesting that a UbL–UIM interaction specific to PLIC-2 is involved or that there may be a differential interaction between the PLICs and UIM-containing proteins. Interestingly, in vitro binding assays suggest that PLIC-2 but not PLIC-1 specifically interacts with Epsin1 and 2 and that PLIC-2 may bind Eps15 with

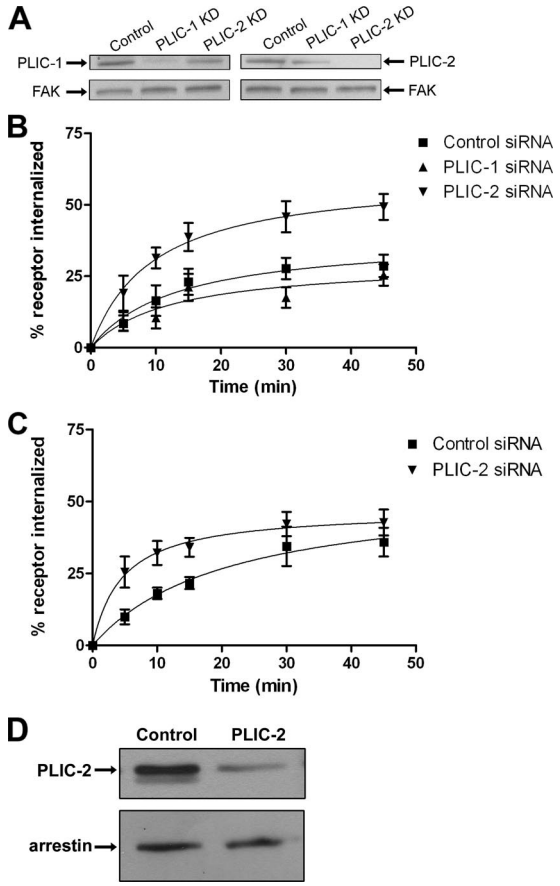


Figure 6. PLIC-2 is required for negative regulation of GPCR endocytosis. (A) Depletion of endogenous levels of PLIC-1 or PLIC-2 was achieved by siRNA transfection. A nonsilencing siRNA was used as control. Levels of each protein were assessed by Western blot on cell lysates. Focal adhesion kinase (FAK) was used as a loading control. (B) PLIC-depleted cells were transfected with FLAG-V2R, fed with M1 anti-FLAG antibody, and AVP-induced receptor endocytosis was measured by flow cytometry as described in A. Graph represents the mean ± SE of six independent experiments. (C) PLIC-depleted cells were transfected with FLAG-β2AR and processed as described in B. Graph represents the mean ± SE of three independent experiments. (D) Cell lysates from HeLa cells transfected with control or PLIC-2 siRNA were analyzed for arrestin expression levels by Western Blot. Shown is a representative experiment.

a higher affinity than PLIC-1. It is still possible that PLIC-2–specific interactions with other unidentified UIM-containing proteins underlie the endocytic function of PLIC-2 in vivo, given that both Eps15 and Epsin are also involved in the internalization of other membrane cargo such as TfR (Vanden Broeck and De Wolf, 2006) and EGFR (Sigismund *et al*., 2005; Vanden Broeck and De Wolf, 2006), and we observe that PLIC-2 specifically regulates GPCR endocytosis with no effect on TfR and EGFR. Given that Epsin and Eps15 function as cargo adaptors for the clathrin pathway, further work remains to fully understand the functional implication of these associations with PLIC-2 during GPCR recruitment to CCPs.

Despite the significant delay in ligand-induced endocytosis of two GPCRs, neither TfR nor EGFR internalization was affected by overexpression of either PLIC-1 or PLIC-2. The molecular basis for this cargo specificity is unclear. We have previously shown that TfR can be internalized via CCPs distinct to those containing GPCRs (Cao *et al*., 1999; Puthenveedu and von Zastrow, 2006). One possibility, therefore, is that PLIC-2 regulation of GPCR clustering rate may function at an early step of the mechanism contributing to the selective concentration of GPCRs in distinct CCP subsets (Cao *et al*., 1999; Mundell *et al*., 2006; Puthenveedu and von Zastrow, 2006).

A further key distinction between GPCRs, the TfR and the EGFR is the dependence on arrestins for internalization. Arrestin 3 (βARR-2) itself undergoes ligand-mediated ubiquitination, which has been shown to be required for β2AR internalization (Shenoy *et al*., 2001). We did not observe any inhibitory effects of PLIC-2 expression on the rate of arrestin-3 translocation from the cytosol to the plasma membrane, or on cellular levels of arrestin, suggesting that PLIC-2 does not affect arrestin expression or its ability to associate with ligand-activated receptors. We did, however, observe that PLIC-2 retards the subsequent localization of GPCRs and arrestins to CCPs. This suggests that PLIC-2 specifically controls the rate at which receptor–arrestin complexes associate with the clathrin-associated coat structure. PLIC-2 could mediate this function by regulating the availability of a UIM-containing protein (such as Eps15 and/or Epsin) that facilitates cargo recruitment into CCPs. TIRF microscopy detected a small fraction of cellular PLIC-2 that associated transiently with CCPs, but the majority of PLIC-2 was present in the cytoplasm (Supplemental Videos S3 and S4). Thus, it remains to be determined whether PLIC-2 regulates GPCR-arrestin recruitment locally, by interacting with individual CCPs, or more globally by interacting with the cellular pool of key endocytic protein(s) outside of CCPs. The proposed model would not require PLIC-2 localization at the plasma membrane or in CCPs to exert its functional effects. TIRF and confocal images indicate that PLIC-2 can localize to multiple compartments, and do not exclude the possibility that the site of PLIC-2 action could be in the cytosol and/or at the plasma membrane. Given that the UBA deletion mutant, known to prevent formation of PLIC-enriched cytoplasmic aggregates, also inhibited receptor internalization, suggests that these PLIC-containing structures are not required for PLIC-2 regulation of GPCR endocytosis.

Agonist-induced internalization of GPCRs is well known to contribute to regulating GPCR signaling by promoting rapid signal desensitization, or by activating signaling pathways after internalization. Therefore, by regulating the rate of GPCR endocytosis, PLIC-2 in turn could contribute to regulating the temporal and spatial dynamics of cell signaling from these receptors. It is interesting to note that PLIC-1 specifically regulates signaling from G_i-coupled receptors (N′Diaye and Brown, 2003), whereas the V2R and β2AR used in this study are G_s-coupled receptors. However, we have observed similar inhibitory effects on endocytosis of a G_i-coupled receptor, the delta-opioid receptor, by PLIC-2 overexpression (data not shown), suggesting a broader scope for PLIC-2 function in GPCR endocytosis. Our results also provide support for the idea that the kinetics of regulated endocytosis are exquisitely regulated by intrinsic cellular mechanisms that either delay or promote cargo recruitment in to CCPs via PLIC-2 levels (this study), and/or the dynamics of the GPCR-containing CCP via regulation of the residency time that GPCRs remain in CCPs (Puthenveedu and von Zastrow, 2006).

Overall, these studies identify an unprecedented role for the ubiquitin-like protein PLIC-2 in negatively regulating GPCR recruitment into CCPs, and they illustrate that a balance of both positive and negative cellular mechanisms

exist which could tightly regulate GPCR signaling and membrane trafficking.

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