The ubiquitin-related protein PLIC-1 regulates heterotrimeric G protein function through association with Gβγ

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PLIC-1, a newly described ubiquitin-related protein, inhibited both Jurkat migration toward SDF-1α and A431 wound healing, but the closely related PLIC-2 did not. PLIC-1 prevented the SDF-1α–induced activation of phospholipase C, decreased ligand-induced internalization of SDF-1α receptor CXCR4 and inhibited chemotaxis signaled by a transfected Gi-coupled receptor. However, PLIC-1 had no effect on Gs-mediated adenylyl cyclase activation, and inhibited only the Gβγ-dependent component of Gq-initiated increase in $[Ca^{2+}]_i$, which is consistent with selective inhibition of Gβγ function. PLIC-1 colocalized with G proteins in lamellae and pseudopods, and precipitated Gβγ in pull downs. Interaction with Gβγ did not require PLIC-1's ubiquitin-like or ubiquitin-associated domains, and proteasome inhibition had no effect on SDF-1α activation of phospholipase C, indicating that PLIC-1's inhibition of Gβγ did not result from effects on proteasome function. Thus, PLIC-1 inhibits Gi signaling by direct association with Gβγ; because it also interacts with CD47, a modulator of integrin function, it likely has a role integrating adhesion and signaling components of cell migration.

Introduction

Chemotaxis requires signaling cross-talk between chemoattractant receptors and the cytoskeleton. Many chemoattractants are recognized by G protein–coupled receptors (GPCRs), sharing the typical structural motif of seven membrane-spanning helices, often signaling through pertussis toxin (PTX)-sensitive heterotrimeric Gi proteins. Chemokine binding to the receptor promotes the release of GDP and binding of GTP to Gαi, leading to the dissociation of Gβγ from the heterotrimeric complex. The released Gβγ can interact with effectors including lipases, kinases, and ion channels required for migration (Thelen, 2001).

To migrate in response to a chemotactic signal, cells need to modulate their adhesive properties in a regulated manner. This involves integrin activation, which can in turn be modulated by association with membrane partners such as tetraspannins, growth factor receptors, or CD47 (Brown, 2002). CD47 is a ubiquitous integral membrane glycoprotein, which is physically and functionally associated with integrins αvβ3, α2β1, αIIbβ3, and α4β1. CD47 ligation has been shown to activate PTX-sensitive G proteins, suggesting a mechanism through which CD47 might regulate migration (Brown and Frazier, 2001).

PLIC-1 and PLIC-2 are two closely related proteins originally identified through their interaction with the cytoplasmic tail of CD47. Sequence analysis reveals ubiquitin-like (Ubq) domains in the amino termini of both proteins, and a ubiquitin-associated (Uba) domain in each carboxy terminus. The region between the Ubq and the Uba domains contains several Sti1 motifs (Kaye et al., 2000) of unknown function. It is this internal region that contains CD47 binding sites. Despite high homology between the two proteins, PLIC-1 binds more tightly to CD47 than PLIC-2, perhaps because it has two internal repeats that interact with CD47 (Wu et al., 1999).

A connection between PLICs and both actin cytoskeleton and intermediate filaments suggested that PLICs may participate in CD47 regulation of adhesion and migration (Wu et al., 1999). Here, we have investigated the role of the PLICs in cell migration and have found that PLIC-1, but not PLIC-2, inhibits cell migration. Surprisingly, this regulation occurs through effects on Gi signaling rather than directly

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on integrin function. Thus, PLIC-1 is involved in communication between integrins and GPCRs and likely is a molecular component of the mechanism through which CD47 regulates cell motility and Gi signal transduction.

Results

PLIC-1, but not PLIC-2, inhibits SDF-1α–induced chemotaxis of Jurkat T cells and serum-induced migration of A431 epithelial cells

To investigate any potential role for PLIC-1 or PLIC-2 in cell migration, we created Jurkat cell lines that stably express PLIC-1 (JPLIC-1), PLIC-2 (JPLIC-2), or a neomycin resistance gene alone (JC). We studied SDF-1α–induced chemotaxis of these cell lines, using a transwell assay. As shown in Fig. 1 A, JPLIC-1 migrated more poorly than JC, whereas JPLIC-2 did not show any significant decrease in migration. The difference in effect on migration was true for multiple independently derived clones of both PLIC-1 and PLIC-2 expressors. Expression level of the SDF-1α receptor CXCR4 was comparable for all cell lines (not depicted), and expression levels of transfected PLIC-1 and PLIC-2 also were comparable (Fig. 1 A, inset). Thus, PLIC-1, but not PLIC-2, inhibited SDF-1α–induced chemotaxis of Jurkat T cells. As expected, because CXCR4 is a Gi-coupled receptor (Chen et al., 1998), PTX completely inhibited Jurkat migration in response to SDF-1α (Fig. 1 C).

To evaluate whether PLIC-1 inhibition of migration also occurred in another cell type, we performed an in vitro wound healing assay on A431 epithelial cells stably transfected with empty vector, PLIC-1 or PLIC-2 (Wu et al., 1999). As shown in Fig. 1 B, PLIC-1, but not PLIC-2, transfectants migrated less than the controls. Thus, PLIC-1 also inhibited migration of A431 epithelial cells, although to a lesser extent than its inhibition of Jurkat migration to SDF-1α. Because the SDF-1α receptor CXCR4 signals exclusively through a PTX-sensitive Gi heterotrimer, we reasoned that the smaller effect of PLIC-1 on A431 migration might result from recruitment of Gi-independent mechanisms for stimulating migration in the wound healing assay, perhaps from growth factors in the cell culture medium. PTX pretreatment partially inhibited migration of A431 cells into the wound (Fig. 1 C), indicating that migration of A431 cells in this assay is only partly dependent on Gi signaling. This suggests that PLIC-1 affects Gi-dependent cell migration, but not cell migration initiated by other pathways.

PLIC-1 blocks SDF-1α–induced PLCβ activation

To test whether PLIC-1 directly affected Gi signaling, we assessed the ability of PLIC-1 to alter $[Ca^{2+}]_i$ responses. As shown in Fig. 2 A, whereas SDF-1α induced an increase in $[Ca^{2+}]_i$ in both JC and JPLIC-2, JPLIC-1 was unable to mount any increase in $[Ca^{2+}]_i$ in response to this agonist. This difference between the effects of PLIC-1 and PLIC-2 on SDF-1α–induced calcium also was reproduced in independently derived clones. PTX completely blocked SDF-1α–induced $[Ca^{2+}]_i$ in JC, confirming its dependence on Gi signaling (unpublished data). In contrast, PLIC-1 had no
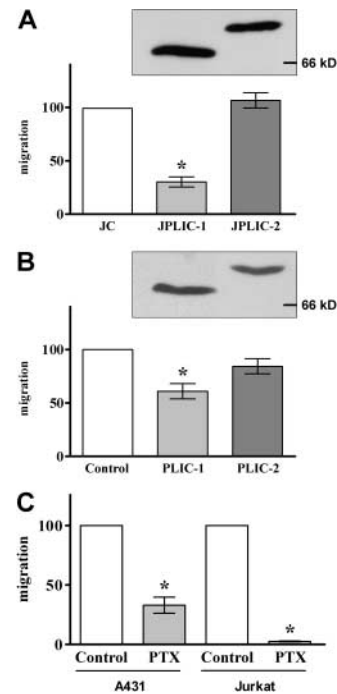

Figure 1. **PLIC-1 but not PLIC-2, inhibits Gi-dependent migration of Jurkat T cells and A431 epithelial cells.** (A) Jurkat T cells transfected with PLIC-1 (JPLIC-1), PLIC-2 (JPLIC-2), or empty vector (JC) were loaded in the upper chamber of a transwell and allowed to migrate to the lower chamber containing SDF-1α. 5 h later, cells that had migrated through the filters into the lower compartment were counted. Samples were performed in triplicate. Results indicate migration relative to the controls (100%) and represent the mean of at least three experiments ± SEM; the asterisk indicates P < 0.05 between control cells and PLIC-1 transfectants. (Inset) Protein expression of myc-tagged PLIC-1 or PLIC-2 shown over the relevant bar was assessed by anti-myc Western blot in total cell lysates from JPLIC-1 or JPLIC-2. PLIC-2 is ∼5 kD larger than PLIC-1. (B) A confluent monolayer of A431 epithelial cells transfected with PLIC-1, PLIC-2, or empty vector was wounded. The distance between wound edges was measured with a micrometer at initiation of wounding and 5 h later, and the distance covered by migrating cells was calculated. Results are expressed as percentage of wound closure relative to vector-transfected controls, and represent the mean of three experiments ± SEM; the asterisks indicate P <0.05. (Inset) Protein expression of PLIC-1 and PLIC-2 in the transfectants was assessed as described above. (C) Before the migration assays described in A and B, control cells were pretreated with PTX. Results represents three independent determinations ± SEM; the asterisks indicate P < 0.05.

significant effect on the $[Ca^{2+}]_i$ increase induced by cross-linking the T cell antigen receptor (Fig. 2 C), which is dependent on tyrosine kinase rather than heterotrimeric G protein signaling (Mustelin and Tasken, 2003).

Because calcium release from intracellular stores in response to heterotrimeric G protein activation is a consequence of IP3 generation by PLCβ (Rhee, 2001), we measured intracellular levels of IP3 after SDF-1α addition in both JC and JPLIC-1. As shown in Fig. 2 B, IP3 increased 30 s after SDF-1α addition to JC, but remained at baseline in JPLIC-1. Because PLCβ2 is a direct effector of Gβγ (Rhee, 2001), we verified that PLIC-1 inhibition was not due to alterations in expression of either Gβ or PLCβ2 protein in JPLIC-1 (Fig. 2 D). Thus, PLIC-1 expression
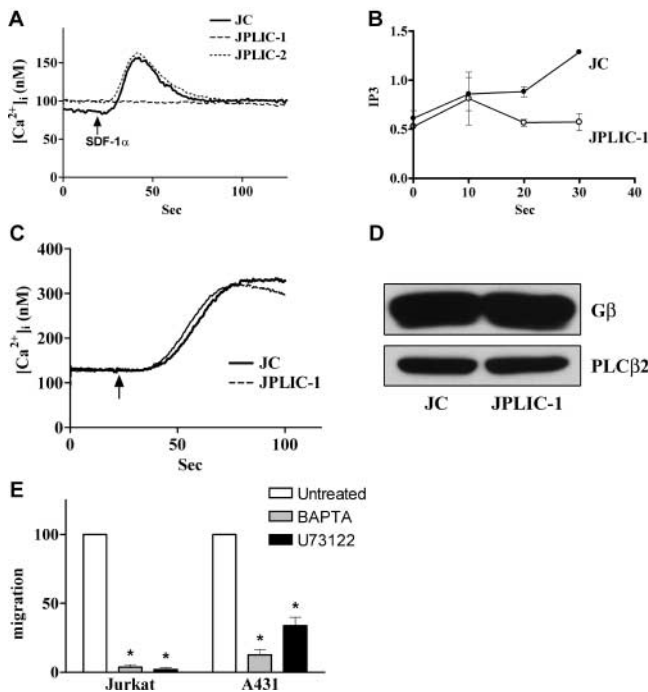
Figure 2.   **PLIC-1 inhibits PLC activation in response to SDF-1α, but not after CD3 ligation.** (A) Fura-2–loaded JC, JPLIC-1, or JPLIC-2 were stimulated with SDF-1α. Intracytoplasmic calcium concentration was determined from fluorescence ratios. A representative experiment of three is shown. (B) JC or JPLIC-1 cells were stimulated with SDF-1α for the indicated times and the amount of IP3 was determined using a radioreceptor assay. A representative experiment of three ± SEM is shown. (C) Fura-2–loaded JC or JPLIC-1 were labeled with anti-CD3 antibody before addition of goat anti–mouse secondary antibody for cross-linking (arrow). A representative experiment of three is shown. (D) Protein expression level of PLCβ2 and Gβ in JC and JPLIC-1 cell lysates was assessed by Western blot using rabbit polyclonal antibodies. (E) Before migration assays, untransfected Jurkat or A431 epithelial cells were pretreated with the calcium chelator BAPTA or the PLC inhibitor U73122. Cell migration was assessed as in Fig. 1. This graph represents the mean of three independent determinations ± SEM; the asterisks indicate P < 0.05.

Figure 3.   **PLIC-1 inhibits Gi and Gq, but not Gs signaling.** JC, JPLIC-1, and JPLIC-2 cells were transiently transfected with Gi-, Gs-, or Gq-coupled GPCRs and tested for related functions. (A) Cells expressing the Gi-coupled receptor Ro2 were loaded in the upper chamber of a transwell apparatus and tested for migration in response to spiradoline. Migrating cells recovered from the bottom chamber were counted and migration of JPLIC-1 and JPLIC-2 cells expressed relative to JC (100%). This graph represents the mean ± SEM of three independent experiments; the asterisk indicates P < 0.05. (B) Isoproterenol-induced cAMP production was measured in JC, JPLIC-1, and JPLIC-2 cells transiently expressing the β2 adrenergic receptor. Data represent the mean ± SEM of three independent experiments. (C) Changes in $[Ca^{2+}]_i$ in response to carbachol were measured in cells transfected with the M3 muscarinic receptor alone or with Pd-like protein. Net calcium increase in JC cells was normalized to 100%, and increase from JPLIC-1 and JPLIC-2 cells determined relative to JC. This graph represents the mean ± SEM of at least five independent experiments; the asterisks indicate P < 0.05.

blocked SDF-1α–induced PLCβ activation. Because PLC activation by CXCR4 was completely inhibited by PTX, and the Gαi subunit does not interact with PLCβ (Rebecchi and Pentyala, 2000), these data demonstrate that PLIC-1 interferes with Gβγ activation of PLCβ2.

To determine whether PLCβ-mediated increase in $[Ca^{2+}]_i$ was required for migration of Jurkat or A431, we examined migration in cells treated with the intracellular Ca^{2+} chelator BAPTA or with the PLC inhibitor U73122. Both BAPTA and U73122 prevented chemotaxis, suggesting a role for PLC in migration of these cells (Fig. 2 E). Thus, PLIC-1 inhibition of SDF-1α–induced PLCβ activation likely contributes to its inhibition of migration.

PLIC-1 inhibits Gi- and Gq-, but not Gs-coupled signaling
To determine whether PLIC-1 affected migration mediated through Gi-coupled receptors other than CXCR4, JPLIC-1, JPLIC-2, and JC were transiently transfected with the Gi-coupled receptor activated solely by a synthetic ligand (RASSL) Ro2, which is similar to κ-opioid receptors but
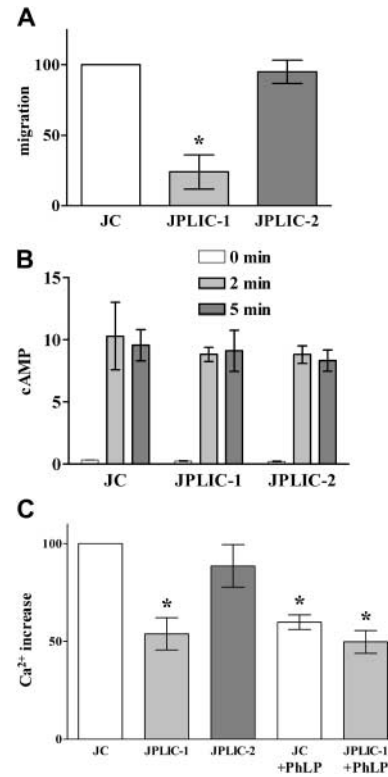
binds spiradoline rather than an opioid ligand (Coward et al., 1998). JC and JPLIC-2 transfected with this receptor migrated in response to spiradoline, but JPLIC-1 cells did not (Fig. 3 A), despite equal expression of the transfected RASSL (not depicted). Thus, PLIC-1 inhibits migration through two different Gi-coupled receptors in Jurkat cells.

To determine the specificity of the inhibitory effect of PLIC-1 for Gi signaling, JPLIC-1, JPLIC-2, and JC were transfected with the Gs-coupled β2 adrenergic receptor or the Gq-coupled M3 muscarinic receptor. Gs signaling was determined by measurement of cAMP production after addition of isoproterenol. PLIC-1 had no effect on the Gs-mediated cAMP increase (Fig. 3 B). Gq function was assessed by increase in $[Ca^{2+}]_i$ after addition of carbachol; JPLIC-1 showed about half the response of JC or JPLIC-2 (Fig. 3 C). Because Gq-coupled receptors can activate PLCβ through both Gαq (which activates primarily PLCβ1) and
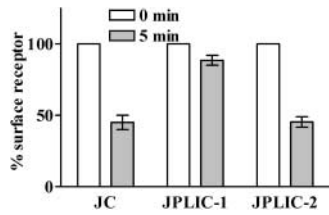
Figure 4. **PLIC-1 blocks SDF-1α–induced CXCR4 endocytosis.**
JC, JPLIC-1, or JPLIC-2 cells were stimulated with SDF-1α for 0 or
5 min. At each time point, cells were washed and labeled with a
CXCR4 antibody and surface expression determined by cytofluorimetry.
A representative experiment ± SEM is shown.

Gβγ (which activates primarily PLCβ2; Rhee, 2001), these
data suggested the possibility that PLIC-1 interfered only
with Gβγ-dependent component of Gq-mediated calcium
increase. To test this hypothesis, the Gβγ inhibitor phosdu-
cin (Pd)-like protein (PhLP; Thibault et al., 1997; Mc-
Laughlin et al., 2002) was cotransfected with the M3 musca-

rinic receptor into JC or JPLIC-1 cells. PhLP expression
partially decreased the calcium response in JC cells, to a
comparable extent as PLIC-1 (Fig. 3 C). In contrast, expres-
sion of PhLP induced no additional decrease in JPLIC-1
cells (Fig. 3 C) despite equal expression of the transfected
protein (not depicted), demonstrating that PLIC-1 and
PhLP act on the same pathway of Gq signal transduction.

To determine if other Gβγ functions were inhibited by
PLIC-1, we tested CXCR4 endocytosis after addition of
SDF-1α, because release and activation of Gβγ from the
heterotrimeric G protein is required for GRK-mediated in-
ternalization of GPCRs (Penn et al., 2000). As shown in Fig.
4, endocytosis of CXCR4 after SDF-1α addition was de-
creased in JPLIC-1 compared with JC or JPLIC-2.

PLIC-1 colocalizes with G proteins and directly interacts with Gβγ

Because PLIC-1 interfered with Gβγ-dependent func-
tions, we looked for a possible association between these

Figure 5. **PLIC-1, not PLIC-2, colocalizes with
Gβ.** Murine lung fibroblasts plated on glass cover-
slips were transfected with myc-tagged (A) PLIC-1
or (B) PLIC-2 and maintained in complete medium
for 24 h. Cells were fixed in formaldehyde and
stained for both myc tag (green) and endogenous
Gβ (red).
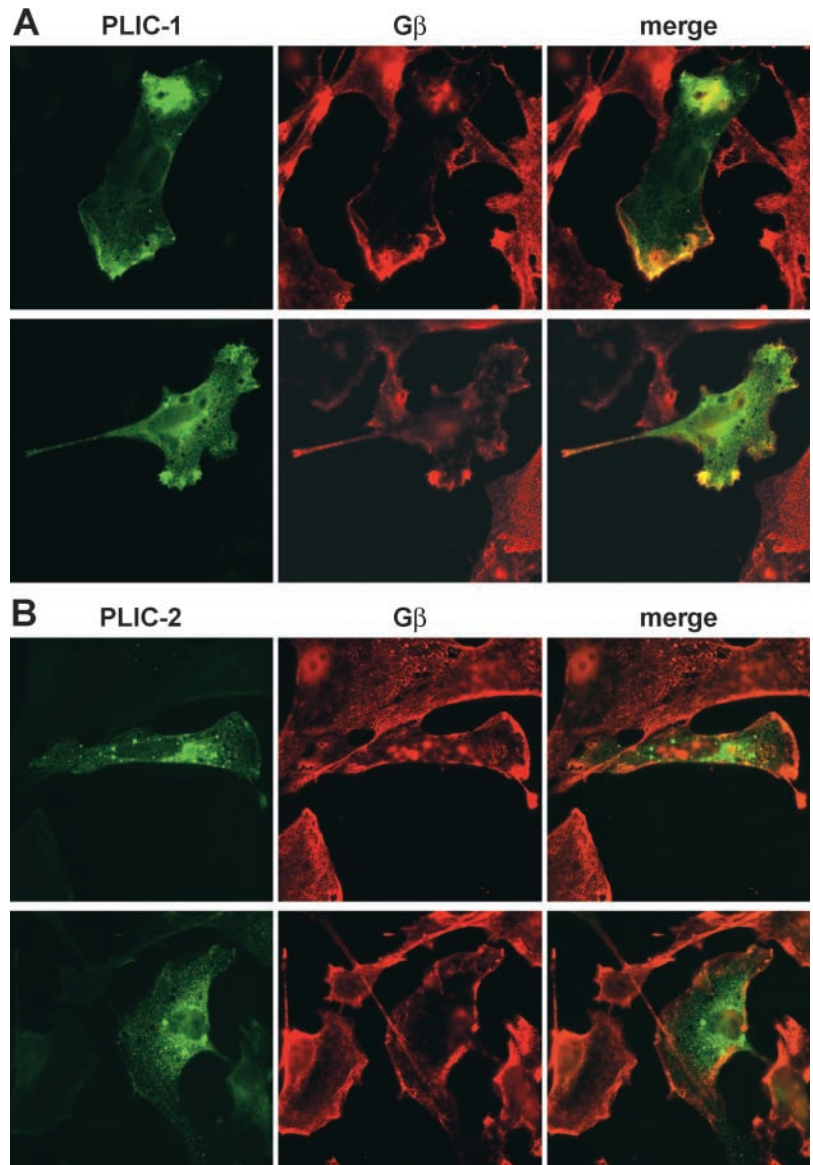
proteins. Immunofluorescence studies were done in fibroblasts because Jurkat cell round morphology interfered with attempts to see specific localization of the proteins. Cells transfected with myc-tagged PLIC-1 or PLIC-2, and stained for both myc and endogenous Gβ showed partial colocalization of PLIC-1 with Gβ at lamella borders and other sites of membrane protrusions (Fig. 5 A). In contrast to PLIC-1, PLIC-2 staining revealed a cytosolic punctate pattern that did not colocalize with Gβ (Fig. 5 B). Thus, a subset of PLIC-1 but not of PLIC-2 colocalized with Gβ.

To examine potential association directly, in vitro pull-down assays using GST–PLIC-1 were performed. PLIC-1 specifically bound Gβ from membranes of Jurkat cells (Fig. 6 A), A431 and HEK-293 cells (not depicted). GST alone and GST–syntaxin 2 did not bind Gβ from any of these cells, and GST–PLIC-1 did not bind lck, which, like Gβγ, is anchored to the plasma membrane by a lipid modification (unpublished data). Unlike Gβ, Gαi did not specifically associate with GST–PLIC-1 even after addition of 100 μM GTP-γ-S to the cell lysate (not depicted), nor did PLCβ2 (Fig. 6 A) or either of the other PLCβ isoforms expressed in Jurkat cells (not depicted). To determine the domain(s) of PLIC-1 required for binding to Gβ, deletion mutants of PLIC-1 were made as shown in Fig. 6 B. Dele-

Figure 7.   **Proteasome inhibition does not block SDF-1α–induced increase in [Ca^{2+}]$_i$.** Lactacystin-treated Jurkat cells were loaded with Fura-2, stimulated with SDF-1α and changes in intracellular calcium recorded. A representative experiment is shown. To assess lactacystin-induced inhibition of the proteasome activity, lysates from vehicle- or lactacystin-treated cells (+LC) were run on SDS-PAGE gel and probed for ubiquitinated proteins with an antiubiquitin Western blot (inset).

tion of both the amino-terminal Ubq and the carboxy-terminal Uba domains did not decrease Gβ binding, demonstrating that the central domain of PLIC-1, which contains the Sti1 motifs, is sufficient for this interaction. Purified Gβγ bound to the same PLIC-1 domain, demonstrating that the interaction is direct (Fig. 6 C). Thus, PLIC-1 binds Gβγ, and the primary site of association is likely to be at membrane protrusions where PLIC-1 preferentially localizes to the plasma membrane.

PLIC-1 inhibition of G protein signaling is independent of effects on proteasome activity

Previous papers have reported the ability of the carboxy-terminal Uba domains of PLIC-related proteins to bind ubiquitinated proteins (Funakoshi et al., 1999; Kleijnen et al., 2000; Mah et al., 2000; Bedford et al., 2001). PLIC family members also bind ubiquitin ligases and proteasome subunits via their amino-terminal Ubq domain (Kleijnen et al., 2000). These interactions are thought to interfere with normal targeting of proteins for proteasome-dependent degradation, resulting in enhanced stability, potentially affecting signaling pathways in which these proteins are involved. However, steady-state levels of Gβ and PLCβ2 were unaffected by PLIC-1 transfection (Fig. 2 D), demonstrating that PLIC-1 inhibition of signaling was not dependent on alteration of the concentration of proximal and distal effectors of the signaling pathway.

To test the possibility that PLIC-1 could affect Gβγ functions by interfering with proteasome activity, we examined the effects of the proteasome inhibitor lactacystin on [Ca^{2+}]$_i$ increase in response to SDF-1α. Lactacystin, which induced a significant accumulation of ubiquitinated proteins in the cells (inset), did not significantly affect SDF-induced calcium changes (Fig. 7). Therefore, we conclude that PLIC-1's inhibition of Gβγ signaling does not require any effect it may have on proteasome activity.

Discussion

The PLICs are a newly recognized family of cytoplasmic and nuclear proteins that have been implicated in a wide variety
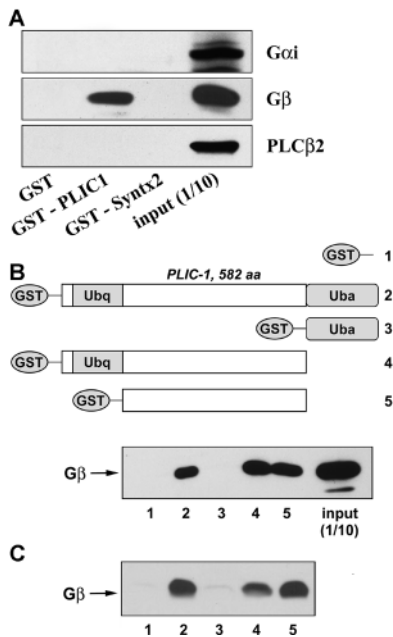

Figure 6.   **PLIC-1 directly binds Gβ.** (A) GST, GST–PLIC-1, or GST–syntaxin2 were incubated with solubilized membranes from Jurkat cells (input). The material from cell lysates that bound to each column was run on SDS-PAGE gel and probed with antibodies against Gβ, Gαi, or PLCβ2. In the right lane, 10% of the starting membrane lysate was analyzed. (B) Full-length PLIC-1 and various deletion mutants were fused to GST and used in the pull-down assay. GST alone (1), full-length PLIC-1 (2), PLIC 1 [aa534–582] (3), PLIC-1 [aa1–538] (4), and PLIC1 [aa100–533] (5) are schematically depicted above the Western blot of material retained by each column and probed for Gβ. (C) Full-length PLIC-1 and the deletion mutants described in B were incubated with purified Gβγ, and material retained by each column probed for Gβ as in B.
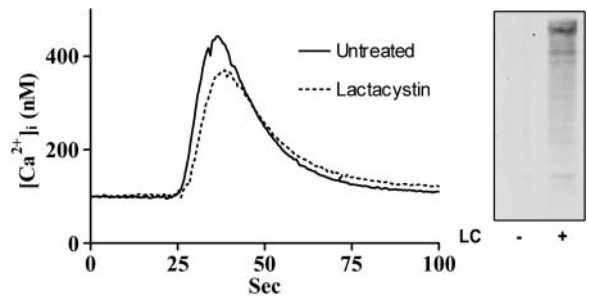
of cellular functions, ranging from inhibition of the cell cycle to stabilization of plasma membrane proteins, and to rearrangements of cytoskeleton (Biggins et al., 1996; Funakoshi et al., 1999; Wu et al., 1999; Bedford et al., 2001). There are four PLIC family members in the mouse and human genomes, and the family is conserved through evolution to the *Saccharomyces cerevisiae* protein dsk2 (Fig. 8). All members of the family have amino-terminal Ubq and carboxy-terminal Uba domains, and the intervening sequences in all metazoan PLICs contain two internal repeats of ~85 aa that contain Sti1 motifs (Kaye et al., 2000). Through yeast two-hybrid approaches, various PLICs have been shown to bind membrane proteins including presenilins, GABA receptors, and CD47; signaling molecules including mTOR (Wu et al., 2002) and cyclin A (Funakoshi et al., 1999); and the chaperonin Stch (Kaye et al., 2000). In addition, both nuclear and cytoplasmic localizations have been reported for different members of the family (Funakoshi et al., 1999; Kleijnen et al., 2000). The Ubq domains of PLIC-2 (Kleijnen et al., 2000; Walters et al., 2002) and of the yeast dsk2 (Funakoshi et al., 2002), bind to components of the proteasome, whereas the Uba domains bind to a variety of ubiquitinated proteins (Kleijnen et al., 2000). Thus, at least some members of the family may be involved in targeting ubiquitinated proteins to the proteasome. However, transfection of PLICs generally inhibits ubiquitin-dependent proteasome degradation (Kleijnen et al., 2000), suggesting that its normal function in protein turnover may be inhibited by overexpression. Although this could explain why expression of the *Xenopus* homologue of human A1u and mouse Ubin, called XDRP-1, could interrupt cell cycle by preventing ubiquitin-mediated degradation of cyclin A (Funakoshi et al., 1999), it would not explain why direct binding of PLIC-1 and its human homologue to several membrane proteins would stabilize these proteins at their membrane location (Mah et al., 2000; Bedford et al., 2001).

Some of this perplexing multitude of functions of the PLICs may derive from the fact that the four isoforms in human and mouse have different functions and different localizations in the cell. However, to date, differences in function or localization among the PLICs have not been reported and only minimally investigated. PLIC-1 and PLIC-2 are the most closely related family members (Fig. 8), yet our data show that they have very different roles in regulating GPCR signaling and very distinct subcellular localizations. PLIC-1, but not PLIC-2, inhibits migration of both A431 epithelial cells and Jurkat T cells. Although we had expected that this would be because of the effects of PLIC-1 on cytoskeleton (Wu et al., 1999), our data suggest that a direct and unique effect of PLIC-1 is on GPCR signaling. Specifically, our data support the hypothesis that PLIC-1 binds the Gβγ subunit of heterotrimeric G proteins and interferes with its normal functions. PLIC-1 precipitated Gβγ in pull-down assays and blocked Gβγ-dependent PLC activation that normally results from SDF-1α binding to CXCR4. Furthermore, PLIC-1 inhibited CXCR4 internalization, which depends on Gβγ release from the G protein after ligand binding to the receptor. In contrast, PLIC-1 did not affect Gαs effector function and likely did not interfere with Gαq function either, suggesting that its effects are specific for Gβγ.
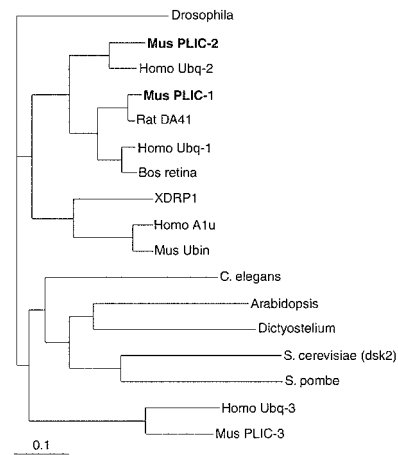

Figure 8. **The PLIC family of proteins.** A phylogenetic tree of multiple members of the PLIC family is depicted. Alignments were performed using ClustalW (31) at the European Bioinformatics Institute web site (http://www.ebi.ac.uk/clustalw) and displayed using TreeView (32). The protein GenBank/EMBL/DDBJ accession numbers are: NP 608344.1 (*Drosophila*); AAF 01366 (Mus PLIC-2); AAG 02474 (Homo Ubq-2); AAF01365 (Mus PLIC-1); BAA92267 (Rat DA41); AAG02473 (Homo Ubq-1); AAK61367 (Bos retina); BAA82642.1 (XDRP1); AAF80171 (Homo A1u); BAB40326 (Mus Ubin); NP 491996.1 (*C. elegans*); NP 179311.1 (*Arabidopsis*); AAF43003.1 (*Dictyostelium*); NP 014003.1 (*S. cerevisiae* dsk2); NP 594159.1 (*S. pombe*); AAF67143 (Homo Ubq-3); and BAC36593.1 (Mus PLIC-3). The PLIC proteins are also called ubiquilins (Ubq). By this nomenclature, human A1u and its mouse orthologue Ubin should be PLIC-4 or ubiquilin-4.

Unfortunately, we were unable to test Gα$_i$ function directly because Gα$_i$ does not decrease cAMP in leukocytes (del Pozo et al., 1995; Elferink and VanUffelen, 1996). However, we did not find evidence for PLIC-1 association with Gαi even under conditions where Gi was activated by GTPγS. In each case, PLIC-2 did not have these functional effects, and, unlike PLIC-1, never localized to plasma membrane, clearly distinguishing the roles for these two closely related proteins. It is of interest that PLIC-2 can associate with membranes in simple fractionation experiments (Wu et al., 1999), suggesting that it may have some role in regulation of signaling at an intracellular membrane or that the cytosolic aggregates in which it can be found (Fig. 5) may be associated with membranes.

We considered several alternative possibilities for the mechanism by which PLIC-1 affected CXCR4 signaling and cell migration. First, we considered that it might affect Ca^{2+} release from cytoplasmic stores in general. However, release of Ca^{2+} by ligation of CD3 was normal, demonstrating that activation of PLCγ and subsequent release of Ca^{2+} stores was unaffected by PLIC-1. We considered that as a protein potentially involved in proteasome function, PLIC-1 might affect the concentration of one or more of the proteins involved in signaling to PLCβ; however, CXCR4 itself, Gβγ, and PLCβ2 expression all were unaffected by PLIC-1. Furthermore, SDF-1α binding to CXCR4 was unaffected by PLIC-1 expression. Finally, we considered that PLIC-1 inhibition of proteasome activity might lead to the signaling aberrations. This is unlikely for several reasons: first, lactacystin, which clearly inhibited proteasome function, had

minimal effect on CXCR4-mediated cytoplasmic Ca^{2+} rise; second, to the extent that it has been studied, PLIC-2 is equivalent to PLIC-1 for proteasome inhibition (Kleijnen et al., 2000); and, finally, we saw no detectable accumulation of ubiquitinated proteins in the PLIC-1 transfectants. Furthermore, although ubiquitination of CXCR4 leads to its degradation, endocytosis of this receptor is independent of its ubiquitination (Marchese and Benovic, 2001), suggesting the effect of PLIC-1 on its internalization is not ubiquitin dependent. Thus, the data are most consistent with PLIC-1–mediated inhibition of Gβγ function independent of its ability to interfere with proteasome activity, and likely through its binding to Gβγ. PLIC-1 has functional similarities with the Pd family of G protein signaling regulators (Schulz, 2001). Pd and its homologue PhLP both bind Gβγ directly with high affinity and inhibit Gβγ-dependent functions by sequestration. Pd, by binding to Gβγ, inhibits β2-adrenergic receptor kinase translocation to the plasma membrane and internalization of the receptor; PhLP blocks internalization of the δ-opioid receptor (Schulz, 2001). Although Pd is expressed exclusively in the retina, PhLP has a broad distribution, and we have found it in Jurkat cells (unpublished data).

Inhibition of Gβγ function is sufficient to account for the ability of PLIC-1 to block cell migration, without postulating additional effects on integrin or cytoskeletal function. Previous papers by the Bourne and Charo groups have established that Gi-mediated chemotaxis absolutely requires release of Gβγ from Gαi, with subsequent activation of Gβγ effectors (Arai et al., 1997; Neptune and Bourne, 1997). Although deletion of the PLCβ2 gene in mice caused primary PMN and lymphocytes to migrate faster (Jiang et al., 1997), in our system, both Ca^{2+} clamping and a PLC inhibitor dramatically decreased cell migration, to about the same extent as PTX. Although reasons for the difference between our results and the knockout are unknown, the fact that there is PTX-sensitive migration in PLCβ2−/− cells suggests that there may be other Gβγ-dependent effector mechanisms important in migration that also are affected by PLIC-1.

These data are the first to demonstrate a significant biological difference between members of the PLIC family. We suggest that the difference in function results, at least in part, from the difference in subcellular localization of PLIC-1 and PLIC-2. Based on what is known of function so far, it appears that PLIC-1 is most closely associated with plasma membrane, PLIC-2 with cytosolic proteasomes, and A1u (called Ubin in the mouse) is likely predominantly expressed in the nucleus. Its ability to associate with the plasma membrane likely is necessary for PLIC-1 to prolong the half-life of GABA receptors and presenilins. It is intriguing that the three membrane proteins (including CD47) with which PLIC-1 has been associated all span the membrane multiple times. It may be that this architecture is important for PLIC-1 interaction.

Finally, it is tantalizing that CD47 is a plasma membrane binding site for PLIC-1 because CD47 ligation has been associated with a nonclassical mechanism for Gi activation (Brown and Frazier, 2001). It may be that CD47 ligation results in loss of association with PLIC-1, releasing an inhibition of Gi signaling, or it may be that activation of Gi signal-

ing through another mechanism recruits PLIC-1 to CD47 at the plasma membrane to restore homeostasis. In either case, the interaction of PLIC-1 with CD47 may represent a novel mechanism for regulation of G protein signaling, and understanding the mechanisms involved in regulating the interaction of these two proteins is likely to reveal additional potential pathways for control of this major plasma membrane signaling pathway.

Materials and methods

Cell culture and transfection
Jurkat cells (E6 clone) were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 0.1% gentamycin. Stably transfected Jurkat cells were maintained under selection with 1.5 mg/ml geneticin (Life Technologies; Reinhold et al., 1997, 1999; Rebres et al., 2001a). A431 epithelial cells and 3656 fibroblasts were maintained in Dulbecco's minimum Essential medium with 10% FCS. A431 stable transfectants were selected with 400 µg/ml geneticin (Wu et al., 1999).

cDNA constructs
cDNA encoding myc or GST-tagged PLIC1 or PLIC-2 were described previously (Wu et al., 1999). GST–syntaxin 2 cDNA was a gift from K. Mostov (University of California San Francisco, San Francisco, CA [UCSF]). HA-tagged M3 muscarinic receptor and Flag-tagged β2 adrenergic receptor cDNAs were provided by H. Bourne (UCSF; Neptune and Bourne, 1997). The cDNA encoding the RASSL Ro2 (Coward et al., 1998) was a gift of B. Conklin (UCSF). Pd-like protein construct was provided by B. Willardson (Brigham Young University, Provo, UT).

To generate deletion mutants of PLIC-1 in fusion with GST, PCR products encompassing the coding regions of PLIC-1 (1–538), PLIC-1 (534–582), and PLIC-1 (100–533) were cloned in frame with the coding region of GST in pGEX-KG vector. The different domains were generated by PCR using the following primers: PLIC-1 (534–582): 5'-GCGAATTCCGCA-GAGTCCAGAAGTCAGATT-3' and 5'-TGCACTCGAGCTATGACGGC-TGGGAACCCAGC-3'; PLIC-1 (1–538): 5'-TGACGGAATTCTTGCCATG-GCCGAGAGCGCAGAGAGCG-3' and 5'-TCGGCCCTCGAGCTATC-AGACTTCTGGACTCTGCAGCTGAGGGTT-3'; PLIC-1 (100–533): 5'-CAGGCGGAATTCGACCGCAAGATAATTCAGCTCAGCAAACA-3' and 5'-TCGGCCCTCGAGCTATCAGACTTCTGGACTCTGCAGCTGAGGGTT-3'. The PCR products were digested with EcoRI/XhoI and cloned into pGEX-KG, and subsequently sequenced to verify that no errors had been introduced during PCR or cloning.

mAbs and reagents
SDF-1α was from PeproTech. Purified Gβγ, PTX, U-73122, BAPTA-AM, lactacystin, GTP–γ-S, isoproterenol, and carbachol were purchased from Calbiochem. Spiradoline was purchased from Sigma-Aldrich. Fura-2–AM was purchased from Molecular Probes. The anti-CD3 mAb (OKT3) was purchased from American Type Culture Collection. Gβ antibodies were purchased from Upstate Biotechnology or BD Transduction Laboratories. Anti-myc antibodies were purchased from Upstate Biotechnology or Invitrogen. Lck, PLCβ2, and Gαi antibodies were purchased from Santa-Cruz Biotechnology, Inc. IP3 and cAMP assay kits were purchased from Amersham Biosciences and BIOMOL Research Laboratories, Inc., respectively.

Cell migration
Chemotaxis of Jurkat T cells in response to SDF-1α was determined using a 24-well plate with 3-µm-pore inserts (BD Biosciences). After filling the lower chamber with medium alone or medium containing 500 ng/ml SDF-1α, 4×10^5 Jurkat T cells (2×10^6/ml in RPMI 1640, 1% FCS) were loaded in the upper chamber. Plates were incubated for 3 h at 37°C in a humidified atmosphere containing 5% CO_2, and cell migration assessed by counting cells in the lower chamber on a hemocytometer. Each experiment was performed in triplicate. The same procedure was used to assess migration of Ro2-transfected Jurkat cells in response to 1 µM spiradoline.

To assess wound healing, A431 cells transfected with empty vector, PLIC-1, or PLIC-2 were grown to confluency. The monolayers were wounded with a pipette tip, washed, and the distance between wound edges measured using a micrometer at specifically marked points along the wound. After 5 h at 37°C in a humidified atmosphere containing 5% CO_2, the distance between wound edges was measured again at the same sites, and the distance covered by migrated cells determined. At least

three different points were used to determine the average distance migrated along the wound edge. In some experiments, confluent monolayers were pretreated overnight with 100 ng/ml PTX, or for 30 min with 4 μM U73122 or 25 μM BAPTA before wounding.

Intracellular calcium measurement

Jurkat T cells (2×10^7 cells/ml) in growth medium were loaded with Fura-2 as described previously (Rebres et al., 2001a,b). Cells were washed once with complete medium and twice with ice-cold Ca^{2+} buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% BSA, and 0.1% glucose), resuspended at 3×10^6 cells/ml in Ca^{2+} buffer, and kept on ice until use. Before stimulation, cells were transferred into cuvettes, prewarmed to 37°C, and placed in a spectrofluorimeter (model F-4500; Hitachi Instruments) after which SDF-1α was added to the stirred cell suspension to a final concentration of 2 μg/ml. To assess the Ca^{2+} response to TCR cross-linking, cells were incubated with OKT3 (American Type Culture Collection) for 30 min on ice, washed twice, and 10 μg/ml anti–mouse IgG was added to the cuvette to cross-link bound antibody in place of SDF-1α. In Jurkat cells transfected with the M3 muscarinic receptor, changes in $[Ca^{2+}]_i$ were measured after stimulation with 100 μM carbachol. Fluorescence was monitored as described previously (Green et al., 1997) and $[Ca^{2+}]_i$ was calculated by the method of Grynkiewicz et al. (1985).

Measurement of intracellular IP3 level

Intracellular IP3 concentration was determined using a radioreceptor assay (Amersham Biosciences). Jurkat T cells were stimulated with 2 μg/ml SDF-1α for various times, and the reaction ended by addition of 0.2 vol ice-cold 20% perchloric acid. The samples were handled, IP3 was measured, and IP3 concentration was calculated exactly as per the manufacturer's instructions.

Measurement of CXCR4 endocytosis

Vector or PLIC-1 transfected Jurkat cells were stimulated with 500 ng/ml SDF-1α for 0 or 5 min. At end point, cells were chilled, washed with cold PBS, and incubated with 10 μg/ml a CXCR4 antibody (Prosciences) for 30 min at 4°C, and subsequently with Alexa 488–coupled anti–mouse IgG (Molecular Probes). Cells were fixed and fluorescence of individual cells was measured by flow cytometry (Coulter Epics).

Immunostaining of PLICs and Gβ

Murine 3656 fibroblasts plated onto coverslips were transfected with myc-tagged PLIC-1 or PLIC-2 and maintained in complete medium for 24 h. Cells were fixed with 3.7% PFA, briefly permeabilized with 0.1% Triton X-100, and stained with Alexa 488–labeled anti-myc (clone 9E10; Upstate Biotechnology) and Alexa 594–labeled anti-Gβ (BD Transduction Laboratories).

Image acquisition

Images shown in Fig. 5 were acquired using a microscope (Axiovert 100TV; Carl Zeiss MicroImaging, Inc.), with a Plan-APOCHROMAT 63X inverted oil objective lens with an NA of 1.40. Cells were fixed and stained with specific antibodies coupled to Alexa 488 or Alexa 594 as described in Immunostaining of PLICs and Gβ, and mounted in Prolong medium (Molecular Probes). Images were acquired with a CCD camera (Micromax; Princeton Instruments) using IPLabs software and subsequently merged using Adobe Photoshop.

GST pull down

GST–PLIC-1 and controls were induced by addition of 0.1 mM IPTG (16 h/RT) to bacterial cultures. Lysates were incubated with GSH-agarose beads (Amersham Biosciences) overnight at 4°C. The beads were washed with a buffer containing 20 mM Hepes, pH 7.4, 25 mM NaCl, 0.5% Triton X-100, 10% glycerol, 0.1% βME, and extensively washed with 20 mM Hepes, pH 7, 150 mM KCl. Cell membranes were prepared by centrifugation of sonicated cells at 100,000 g for 1 h and solubilized in 0.1% Triton X-100 for 2 h at 4°C under constant rotation. Solubilized proteins were separated from insoluble pellet by centrifugation at 15,000 g, and incubated with the GST protein–loaded agarose beads overnight at 4°C. After washing, bead-bound protein was eluted in Laemmli buffer and analyzed by SDS-PAGE and Western blot. To assess potential Gαi binding, the membrane lysate was incubated with 100 μM GTP–γ-S for 15 min at 30°C before incubation with fusion proteins.

To test direct binding of Gβγ, 12 ng of purified Gβγ (Calbiochem) was incubated with the same amount of GST fusion proteins in PBS/0.1% Triton X-100 overnight at 4°C. Bead pellets were washed three times, solubilized in Laemmli buffer and analyzed by SDS-PAGE and Western blot.

Statistical analysis

Data were analyzed by t test.

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