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

Elsa-Noah N'Diaye and Eric J. Brown

Program in Host–Pathogen Interactions, University of California, San Francisco, San Francisco, CA 94143

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 adenyl cyclase activation, and inhibited only the Gβγ-dependent component of Gq-initiated increase in [Ca2+]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 tetraspanins, growth factor receptors, or CD47 (Brown, 2002). CD47 is a ubiquitous integral membrane glycoprotein, which is physically and functionally associated with integrins αβ3, α2β1, α1βb3, 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. 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

Key words: cell migration; CD47; chemokines; migration; signal transduction

Address correspondence to Eric J. Brown, Program in Host–Pathogen Interactions, University of California, San Francisco, Campus Box 2140, 600 16th St., San Francisco, CA 94143-2140. Tel.: (415) 514-0167. Fax: (415) 514-0169; email: ebrown@medicine.ucsf.edu

Abbreviations used in this paper: GPCR, G protein–coupled receptor; Pd, phosducin; PhLP, phosducin-like protein; PTX, pertussis toxin; Uba, ubiquitin-associated; Ubq, ubiquitin-like.
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 (PLIC-1), PLIC-2 (PLIC-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. 1A, 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. 1A, 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. 1C).

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. 1B, 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. 1C), 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 [Ca2+]i responses. As shown in Fig. 2A, whereas SDF-1α induced an increase in [Ca2+]i, in both JC and JPLIC-2, JPLIC-1 was unable to mount any increase in [Ca2+]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 [Ca2+]i in JC, confirming its dependence on Gi signaling (unpublished data). In contrast, PLIC-1 had no significant effect on the [Ca2+]i increase induced by cross-linking the T cell antigen receptor (Fig. 2C), 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. 2B, 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. 2D). Thus, PLIC-1 expression

---

**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 asterisks indicate 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 sequence 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. 2B, 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. 2D). Thus, PLIC-1 expression
PLIC-1 inhibits G\textsubscript{i}i/H9251/H9253 function | N’Diaye and Brown 1159

blocked SDF-1\textsubscript{a}–induced PLC\textsubscript{2} activation. Because PLC activation by CXCR4 was completely inhibited by PTX, and the G\textsubscript{i}i subunit does not interact with PLC\textsubscript{2} (Rebecchi and Pentyala, 2000), these data demonstrate that PLIC-1 interferes with G\textsubscript{i}i/H9251/H9253 activation of PLC\textsubscript{2}.

To determine whether PLC\textsubscript{2}–mediated increase in [Ca\textsuperscript{2+}]\textsubscript{i} was required for migration of Jurkat or A431, we examined migration in cells treated with the intracellular Ca\textsuperscript{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-1\textsubscript{a}–induced PLC\textsubscript{2} 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 Ro2, which is similar to \(\kappa\)-opioid receptors but binds spiradoline rather than an opioid ligand (Coward et al., 1998). JC and JPLIC-2 transfected with this receptor 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 \(\beta\)2 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\textsuperscript{2+}], 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 PLC\textsubscript{B} through both G\textsubscript{q} (which activates primarily PLC\textsubscript{B1}) and
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 phosducin (Pd)-like protein (PhLP; Thibault et al., 1997; McLaughlin et al., 2002) was cotransfected with the M3 muscarinic 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, expression 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 internalization of GPCRs (Penn et al., 2000). As shown in Fig. 4, endocytosis of CXCR4 after SDF-1α addition was decreased 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 functions, we looked for a possible association between these
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, Goi 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. Deletion 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 function by interfering with proteasome activity, we examined the effects of the proteasome inhibitor lactacystin on [Ca2+]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
of cellular functions, ranging from inhibition of the cell cycle to stabilization of plasma membrane proteins, and to re-arrangements 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 \( \sim 85 \) 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 cyttoplasmic localizations have been reported for different members of the family (Funakoshi et al., 1999; Kleijnjen et al., 2000). The Ubq domains of PLIC-2 (Kleijnjen 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 (Kleijnjen 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 (Kleijnjen 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 G\( \beta \)\( \gamma \) subunit of heterotrimeric G proteins and interferes with its normal functions. PLIC-1 precipitated G\( \beta \)\( \gamma \) in pull-down assays and blocked G\( \beta \)\( \gamma \)-dependent PLC activation that normally results from SDF-1\( \alpha \) binding to CXCR4. Furthermore, PLIC-1 inhibited CXCR4 internalization, which depends on G\( \beta \)\( \gamma \) release from the G protein after ligand binding to the receptor. In contrast, PLIC-1 did not affect G\( \alpha \) effect function and likely did not interfere with G\( \alpha \)q function either, suggesting that its effects are specific for G\( \beta \)\( \gamma \).

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); AAF 01365 (Mus PLIC-1); BAA92267 (Rat DA41); AAG 02473 (Homo Ubq-1); AAK61367 (Bos retina); BAA8642.1 (XDRP1); AAF 0171 (Homo A1u); BAB40362 (Mus Ubin); NP 491996.1 (C. elegans); NP 179311.1 (Arabidopsis); AAF 3003.1 (Dictyostelium); NP 014003.1 (S. cerevisiae dsk2); NP 594159.1 (S. pombe); AAF 67143 (Homo Ubq-3); and BAC 63931.1 (Mus PLIC-3). The PLIC proteins are also called ubiquilins (Ubin). By this nomenclature, human A1u and its mouse orthologue Ubin should be PLIC-4 or ubiquilin-4.

Unfortunately, we were unable to test G\( \alpha \)q function directly because G\( \alpha \)q 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 G\( \alpha \)i even under conditions where Gi was activated by GTP\( \gamma \)S. 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 PLC\( \gamma \) 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 PLC\( \beta \); however, CXCR4 itself, G\( \beta \)\( \gamma \), and PLC\( \beta \)2 expression all were unaffected by PLIC-1. Furthermore, SDF-1\( \alpha \) 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 G\(\beta\)\(\gamma\) function independent of its ability to interfere with proteasome activity, and likely through its binding to G\(\beta\)\(\gamma\). PLIC-1 has functional similarities with the Pd family of G protein signaling regulators (Schulz, 2001). Pd and its homologue PhLP both bind G\(\beta\)\(\gamma\) directly with high affinity and inhibit G\(\beta\)\(\gamma\)-dependent functions by sequestration. Pd, by binding to G\(\beta\)\(\gamma\), inhibits \(\beta_2\)-adrenergic receptor kinase translocation to the plasma membrane and internalization of the receptor; PhLP blocks internalization of the \(\delta\)-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 G\(\beta\)\(\gamma\) 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 G\(\beta\)\(\gamma\) from G\(\alpha_i\), with subsequent activation of G\(\beta\)\(\gamma\) effectors (Arai et al., 1997; Neptune and Bourne, 1997). Although deletion of the PLC\(\beta\)2 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 PLC\(\beta\)2/–/– cells suggests that there may be other G\(\beta\)\(\gamma\)-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 Alu (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 a loss of association with PLIC-1, releasing an inhibition of Gi signaling, or it may be that activation of Gi signaling 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 genetin (Life Technologies; Reinhold et al., 1997, 1999; Rebres et al., 2001a). A431 epithelial cells and 3636 fibroblasts were maintained in Dulbecco’s minimum Essential medium with 10% FCS. A431 stable transfectants were selected with 400 \(\mu\)g/ml genetin (Wu et al., 1999).

**cDNA constructs**

cDNA encoding myc or GST-tagged PLIC1 or PLIC-2 were described previously (Wu et al., 1999). GST–syntxin 2 cDNA was a gift from K. Moscov (University of California San Francisco, San Francisco, CA [UCSF]). HA-tagged M3 muscarinic receptor and Flag-tagged \(\beta_2\) 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′-GCCAATTCGCGACGATGTGACAT-3′ and 5′-TGGCACTGACCGTCGAGGCTG-3′; PLIC-1 (1–538): 5′-TCGAGAGGCGTGATGACGTGACG-3′ and 5′-TCGAGAGGCGTGATGACGTGACG-3′; and 5′-TCGAGAGGCGTGATGACGTGACG-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-1a was from PeproTech. Purified G\(\beta\)\(\gamma\), PTX, U-73122, BAPTA-AM, lactacystin, GTP–\(\gamma\)-S, isoprotenerol, and carbobach 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. G\(\beta\)\(\gamma\) antibodies were purchased from Upstate Biotechnology or BD Transduction Laboratories. Anti-myc antibodies were purchased from Upstate Biotechnology or Invitrogen. Lck, PLC\(\beta\)2, and G\(\alpha_i\) antibodies were purchased from Santa-Cruz Biotechnology, Inc. IP3 and cAMP assay kits were purchased from Amerham Biosciences and BIOMOL Research Laboratories, Inc., respectively.

**Cell migration**

Chemotaxis of Jurkat T cells in response to SDF-1a was determined using a 24-well plate with 3-\(\mu\)m-pore inserts (BD Biosciences). After filling the lower chamber with medium alone or medium containing 500 ng/ml SDF-1a, 4 \(\times\) 10⁴ Jurkat T cells (2 \(\times\) 10⁵/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 \(\mu\)M spiradoline.

To assess wound healing, A431 cells transfected with empty vector, PLIC-1, or PLIC-2 were grown to confluency. The monolayers were performed in triplicate. The same procedure was used to assess migration of Ro2-transfected Jurkat cells in response to 1 \(\mu\)M 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 BAPTA before wounding.

Intracellular calcium measurement
Jurkat T cells (2 × 10⁶ cells/ml) in growth medium were loaded with Fura-2 as described previously (Rebers et al., 2001a,b). Cells were washed once with complete medium and twice with ice-cold Ca²⁺ buffer (25 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% BSA, and 0.1% glucose), resuspended at 3 × 10⁶ cells/ml in Ca²⁺ buffer, and kept on ice until use. Before stimulation, cells were transferred into cuvettes, prewarmed to 37°C, and placed in a spectrophotometer (model F-4500; Hitachi Instruments) after which SDF-1α was added to the stirred cell suspension to a final concentration of 2 μM. To assess the Ca²⁺ 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 antibody in place of SDF-1α. In Jurkat cells transfected with the M3 muscarinic receptor, changes in [Ca²⁺], were measured after stimulation with 100 μM carbachol. Fluorescence was monitored as described previously (Green et al., 1997) and [Ca²⁺], 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 in 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 Gß
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-Gß (BD Transduction Laboratories).

Image acquisition
Images shown in Fig. 5 were acquired using a microscope (Axiovert 100TV; Carl Zeiss Microlmaging, Inc.), with a Plan-APochromatic 63× 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 Gß, and mounted in Prolong medium (Molecular Probes). Images were acquired with a CCD camera (Micromax; Princeton Instruments) using IPLab 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 GST-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% BME, 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, 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–γS 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.

The authors thank Mark von Zastrow and Dean Sheppard for many useful suggestions, and Henry Bourne and Barry Willardson for suggestions and reagents. We thank Hiroshi Morisaki for help with microscopy, and Mette Johansen, Robert Rebres, and Bryant MacLauglin for critical reading of the manuscript.

This work was supported by grants GM38330 and AI24674 from the National Institutes of Health to E.J. Brown.

Submitted: 25 July 2003
Accepted: 10 October 2003

References
Arai, H., C.L. Tsou, and I.F. Charo. 1997. Chemotaxis in a lymphocyte cell line transfected with C-C chemokine receptor 2B: evidence that directed migration is mediated by betagamma dimers released by activation of Galpha-coupled receptors. Proc. Natl. Acad. Sci. USA. 94:14495–14499.
Bedford, F.K., J.T. Kirtler, E. Muller, P. Thomas, J.M. Uren, D. Merlo, W. Wisden, A. Teiller, T.G. Smart, and S.J. Moss. 2001. GABAR receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plec-1. Nat. Neurosci. 4:908–916.
Biggins, S., I. Ivanovska, and M.D. Rose. 1996. Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. J. Cell Biol. 133:1331–1346.
Brown, E.J. 2002. Integrin-associated proteins. Curr. Opin. Cell Biol. 14:603–607.
Brown, E.J., and W.A. Frazier. 2001. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol. 11:130–135.
Chen, W.J., C. Jayawickreme, C. Watson, L. Wolfe, W. Holmes, R. Ferris, S. Ar, W. Dallas, G. Chen, L. Boone, et al. 1998. Recombinant human CXC-chemokine receptor-4 in melanophores are linked to Gß protein: seven transmembrane coreceptors for human immunodeficiency virus entry into cells. Mol. Pharmacol. 53:177–181.
Coward, P., H.G. Wada, M.S. Falk, S.D. Chan, F. Meng, H. Akil, and B.R. Conklin. 1998. Controlling signaling with a specifically designed Gi-coupled receptor. Proc. Natl. Acad. Sci. USA. 95:352–357.
del Pozo, M.A., P. Sanchez-Mateos, M. Nieto, and F. Sanchez-Madrid. 1995. Chemokines regulate cellular polarization and adhesion receptor redistribution during lymphocyte interaction with endothelium and extracellular matrix. Involvement of CAM signaling pathway. J. Cell Biol. 131:495–508.
Elferink, J.G., and B.E. VanUffelen. 1996. The role of cyclic nucleotides in neurotrophin migration. Gen. Pharmacol. 27:387–393.
Funakoshi, M., S. Geley, T. Huu, T. Nishimoto, and H. Kobayashi. 1999. Identification of XDRP1; a Xenopus protein related to yeast Dsk2p binds to the N-terminus of cyclin A and inhibits its degradation. EMBO J. 18:5009–5018.
Funakoshi, M., T. Sasaki, T. Nishimoto, and H. Kobayashi. 2002. Budding yeast Dsk2p is a polyubiquitin-binding protein that can interact with the proteasome. Proc. Natl. Acad. Sci. USA. 99:745–750.
Green, J.M., A.D. Schreiber, and E.J. Brown. 1997. Role for a glycan phosphoinositol anchor in Fc γ receptor synergy. J. Cell Biol. 139:1209–1217.
Gryniewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260:3440–3450.
Jiang, H., Y. Kuang, Y. Wu, W. Xie, M.I. Simon, and D. Wu. 1997. Roles of phospholipase C beta2 in chemoattractant-elicted responses. Proc. Natl. Acad. Sci. USA. 94:7971–7975.
Kaye, F.J., S. Modi, I. Ivanovska, E.V. Koonin, K. Thress, A. Kubo, S. Kornbluth, and M.D. Rose. 2000. A family of ubiquitin-like proteins binds the ATPase domain of Hsp70-like Sos. FEBS Lett. 467:348–355.
Kleijn, M.A., E.H. Shih, P. Zhou, S. Kumar, R.E. Soccio, N.L. Kedeska, G. Gill, and P.M. Howley. 2000. The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. Mol. Cell. 6:409–419.
Mah, A.L., G. Perry, M.A. Smith, and M.J. Monteiro. 2000. Identification of ubiquitin, a novel presenilin interactor that increases presenilin protein accumulation. J. Cell Biol. 151:847–862.
Marchese, A., and J.L. Benovic. 2001. Agonist-promoted ubiquitination of the Gαi binding, the membrane lysate was incubated with 100 μM GTP–γS for 15 min at 30°C before incubation with fusion proteins.
protein-coupled receptor CXCR4 mediates lysosomal sorting. *J. Biol. Chem.* 276:45509–45512.

McLaughlin, J.N., C.D. Thulin, S.M. Bray, M.M. Martin, T.S. Elton, and B.M. Willardson. 2002. Regulation of angiotensin II-induced G protein signaling by phosducin-like protein. *J. Biol. Chem.* 277:34885–34895.

Mustelin, T., and K. Tasken. 2003. Positive and negative regulation of T-cell activation through kinases and phosphatases. *Biochem. J.* 371:15–27.

Neptune, E.R., and H.R. Bourne. 1997. Receptors induce chemotaxis by releasing the betagamma subunit of Gi, not by activating Gq or Go. *Proc. Natl. Acad. Sci. USA.* 94:14489–14494.

Penn, R.B., A.N. Pronin, and J.L. Benovic. 2000. Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc. Med.* 10:81–89.

Rebecchi, M.J., and S.N. Pentyala. 2000. Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol. Rev.* 80:1291–1335.

Rebres, R.A., J.M. Green, M.I. Reinhold, M. Ticchioni, and E.J. Brown. 2001a. Membrane raft association of CD47 is necessary for actin polymerization and protein kinase C theta translocation in its synergistic activation of T cells. *J. Biol. Chem.* 276:7672–7680.

Rebres, R.A., L.E. Vaz, J.M. Green, and E.J. Brown. 2001b. Normal ligand binding and signaling by CD47 (integrin-associated protein) requires a long range disulfide bond between the extracellular and membrane-spanning domains. *J. Biol. Chem.* 276:34607–34616.

Reinhold, M.I., F.P. Lindberg, G.J. Kersh, P.M. Allen, and E.J. Brown. 1997. Co-stimulation of T cell activation by integrin-associated protein (CD47) is an adhesion-dependent, CD28-independent signaling pathway. *J. Exp. Med.* 185:1–11.

Reinhold, M.I., J.M. Green, F.P. Lindberg, M. Ticchioni, and E.J. Brown. 1999. Cell spreading distinguishes the mechanism of augmentation of T cell activation by integrin-associated protein/CD47 and CD28. *Int. Immunol.* 11:707–718.

Rhee, S.G. 2001. Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.* 70:281–312.

Schulz, R. 2001. The pharmacology of phosducin. *Pharmacol. Rev.* 43:1–10.

Thelen, M. 2001. Dancing to the tune of chemokines. *Nat. Immunol.* 2:129–134.

Thibault, C., M.W. Sganga, and M.F. Miles. 1997. Interaction of phosducin-like protein with G protein betagamma subunits. *J. Biol. Chem.* 272:12253–12256.

Walters, K.J., M.F. Kleijnen, A.M. Goh, G. Wagner, and P.M. Howley. 2002. Structural studies of the interaction between ubiquitin family proteins and proteasome subunit S5a. *Biochemistry.* 41:1767–1777.

Wu, A.L., J. Wang, A. Zheleznyak, and E.J. Brown. 1999. Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane. *Mol. Cell.* 4:619–625.

Wu, S., A. Mikhailov, H. Kallo-Hosein, K. Harz, K. Yonezawa, and J. Avruch. 2002. Characterization of ubiquilin 1, an mTOR-interacting protein. *Biochim. Biophys. Acta.* 1542:41–56.