Critical events for vasoconstrictor and growth factor signal transduction include stimulation of phospholipase Cγ (PLCγ) and elevation of intracellular calcium. c-Src has been proposed as a common mediator for these signals activated by both G protein-coupled receptors (GPCRs) and tyrosine kinase-coupled receptors (TKRs). Here we show that the GPCR kinase-interacting protein-1 (GIT1) is a substrate for c-Src that undergoes tyrosine phosphorylation in response to angiotensin II (AngII) and EGF in vascular smooth muscle and 293 cells. GIT1 associates with PLCγ via the PLCγ Src homology 2 and 3 domains constitutively, and the interaction is unaltered by AngII and EGF. GIT1 interaction with PLCγ is required for PLCγ activation based on inhibition of tyrosine phosphorylation and calcium mobilization after GIT1 knockdown with antisense GIT1 oligonucleotides. GIT1 interacts with PLCγ via a novel Spa homology domain (SHD) and a coiled-coil domain. Deletion mutation analysis showed that GIT1(SHD) is required for AngII- and EGF-mediated PLCγ activation (measured by phosphorylation of Tyr783 and inositol 1,4,5-trisphosphate formation). We propose that GIT1 is a novel regulator of PLCγ function that mediates PLCγ activation by Src and integrates signal transduction by GPCRs and TKRs.

Several signal transduction events induced by angiotensin II (AngII) binding to the AngII type 1 receptor (AT1R) resemble those stimulated by EGF and PDGF binding to their receptors.

1–3. AngII directly causes cell proliferation and cell hypertrophy of many cell types including vascular smooth muscle cells (VSMC), cardiac fibroblasts, mesangial cells, and macrophages (4–6). AngII stimulates gene transcription (e.g. c-jun and c-fos) (7, 8), induces gene expression of enzymes that produce mediators of inflammation (e.g. NADPH oxidase, phospholipase A2, and phospholipase D) (9, 10) and activates multiple intracellular signaling cascades (e.g. tyrosine kinases, Ca2+-calmodulin, and mitogen-activated protein kinase) (11). AngII-mediated growth effects in VSMC also require the rapid activation of phospholipase Cγ (PLCγ) and mobilization of intracellular calcium (12–14). Early growth response genes induced by AngII such as c-fos and c-myc require calcium mobilization and activation of AP-1-dependent transcription. A key role for tyrosine kinases in AngII-mediated increases in intracellular calcium has been established (7). There is evidence that binding of AngII to the AT1R stimulates tyrosine phosphorylation of several proteins including Shc and GRB-2 prior to activation of members of the Ras family (15). Rapid tyrosine phosphorylation is probably mediated by nonreceptor tyrosine kinases including Src family tyrosine kinases, JAK2 and PYK2 (16–22). Activation of PLCγ requires tyrosine phosphorylation as shown by inhibition of Ins1,4,5P3 formation with inhibitors such as herbimycin A and genistein (13). A specific role for Src was suggested by demonstrating rapid activation of Src in VSMC, inhibition of PLCγ activation with antibodies to Src, and impaired signal transduction in Src−/− cells (12, 16, 23). It is well known that PLCγ1 and PLCγ2 are substrates for members of the Src kinase family (24, 25). Specific to AngII is the fact that AngII-mediated transactivation of the EGF receptor may be mediated by c-Src (22), suggesting important roles for both EGFR and c-Src in AngII-mediated events.

To understand the role of Src kinases in AngII-mediated signal transduction, we have focused on PLCγ tyrosine phosphorylation as a key rapid event. We showed previously that AngII stimulated tyrosine phosphorylation of a ~97-kDa protein (p97) that associated with PLCγ in VSMC (26). AngII-induced tyrosine phosphorylation of both PLCγ and the p97 were dependent on Src (26). To determine the role of p97 in signal transduction mediated by Src and PLCγ, we purified, identified, and sequenced p97 that co-precipitated with PLCγ. Here, we demonstrate that p97 is the same as GIT1, originally characterized as a G protein-coupled receptor kinase-interacting protein (27). Two GIT family members with numerous tissue-specific alternatively spliced isoforms have been identified in mammals and chicken. These include GIT1 (also termed Cat-1 for Coool (cloned out of library)-associated tyrosine-phosphorylated pro-
GIT1 Phosphorylation Regulates PLCγ
49937

Materials and Methods

Mass Spectrometric Analysis—The band corresponding to the 97-kDa protein was cut out of a silver-stained SDS-polyacrylamide gel and digested with trypsin. Four tryptic peptides were analyzed by mass spectrometric microsequence analysis (two showed the same sequence) as described previously (35). The sequences derived from these four peptides showed absolute identity with GIT1.

Cells and Transfection—VSMC were obtained from rat aorta as described (36). HEK293 cells and VSMC were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO2. HEK293 cells were transfected with LipofectAMINE Plus (Invitrogen). VSMC were transfected using the FuGENE 6 Reagent (Roche Applied Science). For cotransfection with AT1R, a ratio of 3:1 was used. After allowing protein expression for 24 h, cells were serum-deprived for 16 h and stimulated with 10 ng/ml EGF or 200 ng AngII. Phosphorylomodulated sense (S), scrambled, or antisense (AS) oligonucleotides corresponding to the GIT1 sequence (S-GIT1, 5′-CTGATGACTGTCTGTCACG-3′; AS-GIT1, 5′-CTGATGACTGTCTGTCACG-3′) were prepared and transfected at 1 μg for 48 h as previously described by our laboratory (37) and then serum-deprived for 16 h and stimulated with 10 ng/ml EGF or 200 ng AngII.

Plasmid Constructs—The mGIT1 expressed sequence tag clone (GenBank accession number AI414223) was purchased and completely sequenced. The clone lacked the last ~200 bp of the C-terminal open reading frame. Therefore, the missing C-terminal fragment was obtained by reverse transcriptase reaction using mouse brain total RNA and specific primers (5′-CTGACGTGAGCATTTAGTATGAGGAC-3′ and 5′-GTCTTAGAGTTCCAGGTCTGGTGGAA-3′, respectively). The full-length mGIT1 (GIT1(wt)) was cloned into the NotI and XbaI site of Xpress-tagged pcDNA3.1 vector (xpress-GIT1(wt)) and the EcoRI and ApaI site of pCMV-Tg2B vector (FLAG-GIT1(wt)). Using PCR, GIT1 (aa 1–635), GIT1 (aa 1–420), GIT1 (aa 420–770), GIT1 (aa 250–770), and GIT1(del-SHD) were cloned into the pCMV-Tg2B vector (FLAG-GIT1 (aa 1–635), FLAG-GIT1 (aa 1–420), FLAG-GIT1 (aa 420–770), FLAG-GIT1 (aa 250–770) FLAG-GIT1(del-SHD)). GIT1(del-C22) mutant was obtained using QuikChangeTM site-directed mutagenesis (Stratagene). Using PCR, GIT1 (aa 1–250) and GIT1 (aa 250–420) were cloned into the BamHI and XhoI site of pGEX-KG (GST-GIT1 (aa 1–250) and GST-GIT1 (aa 250–420)). The insert sequence and reading frame were confirmed by sequencing.

Immunoprecipitation and Immunoblotting—Anti-glutathione S-transferase (GST) monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GIT1 monoclonal antibody was from BD Transduction Laboratories (Lexington, KY). Anti-FLAG M2 and anti-FLAG monoclonal antibodies were from Sigma. Anti-GIT1 antibody monoclonal antibody was from Transduction Laboratories (Lexington, KY), anti-FLAG and anti-HA antibody was from Roche Applied Science. Anti-TyrP70m-PLC was from Cell Signaling (Beverly, MA). For immunoprecipitations, cells were lysed in radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, pH 8.0). Analysis of autoradiograms after immunoblotting was performed by scanning densitometry and processing with NIH Image. Statistical analysis was performed with Student’s t test.

Results

A c-Src-dependent Phosphoprotein That Interacts with PLCγ

Specifically Is GIT1—To define Src kinase substrates that mediate GPCR signal transduction, we characterized proteins rapidly tyrosine-phosphorylated in an Src-dependent manner. Previous data from our laboratory suggested an essential role for c-Src in AT1R signal transduction, especially mediated by PLCγ, which is tyrosine-phosphorylated within 1 min of AngII binding (16, 23, 26, 40). Therefore, we immunoprecipitated proteins from VSMC that associated with PLCγ and characterized proteins that were dependent on Src using both pharmacologic inhibition and Src−/− cells. This analysis yielded a 97-kDa protein (p97) that was tyrosine-phosphorylated in response to AngII (26). p97 was excised from a silver stained SDS-polyacrylamide gel, digested with trypsin, and analyzed by mass spectrometry and microsequence analysis (35). Comparison with the SWISS-PROT protein sequence database entries yielded a complete match with GIT1 previously identified by Premont et al. (27) (Fig. 1A). In growth-arrested VSMC, there was a low level of GIT1 tyrosine phosphorylation, which rapidly and transiently increased by 2.2 ± 0.2-fold in response to AngII after 1 min (Fig. 1, B and C, upper panels, n = 6, p < 0.05). In the absence of AngII, GIT1 was bound to PLCγ as shown by co-precipitation in GIT1 immunoprecipitates (Fig. 1B, bottom panels), and the interaction did not change with AngII. PLCγ also exhibited a low level of tyrosine phosphorylation under control conditions, and AngII increased PLCγ tyrosine phosphorylation by 2.5 ± 0.5-fold at 1 min (Fig. 1B, second panel; n = 6, p < 0.05). Similar results were obtained when PLCγ was immunoprecipitated (Fig. 1C). There was no change in the amount of GIT1 that co-precipitated with PLCγ in response to AngII (Fig. 1C, third panel), indicating that GIT1 protein, already associated with PLCγ under basal conditions, was being phosphorylated. It is well established that AngII activates PLCδ and PLCβ as well as PLCγ (41). Neither PLCδ nor PLCβ bound to GIT1 in VSMC under basal conditions or after AngII stimulation (not shown). The relative expression levels of PLCγ, PLCδ, and PLCβ in VSMC were similar, as assessed by Western blot (not shown).

GIT1 Plays a Key Role in Stimulation of PLCγ Tyrosine Phosphorylation and Calcium Mobilization in VSMC—To show a role for GIT1 in PLCγ function, we used antisense GIT1 oligonucleotides to decrease GIT1 expression in VSMC as pre-
FIG. 1. Identification and cloning of GIT1 and binding of GIT1 to PLCγ. A, the derived tryptic peptides from the 97-kDa protein that immunoprecipitated with PLCγ are shown below the sequence of mouse GIT1. The peptides showed absolute identity with GIT1. B and C, AngII-induced tyrosine phosphorylation of GIT1 and PLCγ. VSMC were stimulated with 200 nM AngII for 1 min. Cell lysates were immunoprecipitated with either GIT1 (B) or PLCγ antibody (C) (IP). Immunoblotting (IB) was performed with anti-phosphotyrosine antibody (4G10, upper two panels). Binding of GIT1 to PLCγ as well as equal loading was assured by stripping and reprobing the membrane with PLCγ or GIT1 antibody (lower two panels). D, sense and antisense GIT1 oligonucleotides were transfected into VSMC as described. Cell lysates were prepared, and GIT1 protein content was measured by Western blot (IB: GIT1). Tyrosine phosphorylation of PLCγ was measured by 4G10 antibody. The specificity of the sense and antisense oligonucleotides was demonstrated by lack of effect on PLCγ expression (IB: PLCγ). There was no effect of 200 nM AngII (1 min) on the interaction between GIT1 and PLCγ, although it is clear that tyrosine phosphorylation of GIT1 increased.
Tides had no significant effect. The decrease in basal PLC induction by AngII (Fig. 1 D). Cellular calcium concentration in response to AngII was 223 nM. The percentage measured calcium mobilization in VSMC using Fura-2. Cells were incubated for 1 min with vehicle, 200 nM AngII, 1 unit/ml thrombin, 10 ng/ml PDGF, or 10 ng/ml EGF. Immunoprecipitation (IP) with PLCγ and immunoblotting (IB) with 4G10 (upper panel) and GIT1 (lower panel) is shown. B, AngII-induced GIT1 tyrosine phosphorylation in rat aorta. Rats were infused for 5 min with AngII as described under "Materials and Methods." Immunoprecipitation (IP) was with PLCγ antibody, and immunoblotting was with the indicated antibodies.

GIT1 was tyrosine-phosphorylated by VSMC agonists. VSMC were incubated for 1 min with vehicle, 200 nM AngII, 1 unit/ml thrombin, 10 ng/ml PDGF, or 10 ng/ml EGF. Immunoprecipitation (IP) with PLCγ and immunoblotting (IB) with 4G10 (upper panel) and GIT1 (lower panel) is shown. B, AngII-induced GIT1 tyrosine phosphorylation in rat aorta. Rats were infused for 5 min with AngII as described under "Materials and Methods." Immunoprecipitation (IP) was with PLCγ antibody, and immunoblotting was with the indicated antibodies.

VSMC: A

VSMC:

IP: PLCγ

pY-GIT1

GIT1

Con Ang II PDGF EGF

IP: PLCγ

IB: 4G10

IB: GIT1

pY-GIT1

GIT1

PLCγ

IB: 4G10

IB: GIT1

IB: PLCγ

AngII

Fig. 2. GIT1 is tyrosine-phosphorylated by VSMC agonists. VSMC were incubated for 1 min with vehicle, 200 nM AngII, 1 unit/ml thrombin, 10 ng/ml PDGF, or 10 ng/ml EGF. Immunoprecipitation (IP) with PLCγ and immunoblotting (IB) with 4G10 (upper panel) and GIT1 (lower panel) is shown. B, AngII-induced GIT1 tyrosine phosphorylation in rat aorta. Rats were infused for 5 min with AngII as described under "Materials and Methods." Immunoprecipitation (IP) was with PLCγ antibody, and immunoblotting was with the indicated antibodies.

GIT1 phosphorylation regulated PLCγ. We initially identified GIT1 as a 97-kDa protein bound to PLCγ after AngII stimulation. However, a more general role for GIT1 in G protein-coupled receptors and tyrosine kinase-coupled receptors has been proposed (34, 45). As shown in Fig. 2A, AngII, thrombin, EGF, and PDGF all stimulated tyrosine phosphorylation of GIT1 in VSMC (from 1.5- to 2.5-fold). We do not believe that AngII-mediated GIT1 phosphorylation was due to transactivation of the PDGF and EGF receptors (46, 47), because AngII stimulated phosphorylation of GIT1 more rapidly and to a greater extent than PDGF and EGF. In addition, inhibiting PDGF and EGF receptors with AG1478 and AG1295 did not alter AngII-stimulated GIT1 phosphorylation, despite inhibiting transactivation (not shown).

To demonstrate that GIT1 is important for AngII function in vivo, we infused rats for 5 min with AngII and analyzed tyrosine phosphorylation of GIT1 (Fig. 2B). In rat aortas, we found that GIT1 bound to PLCγ in both control and agonist-treated aortas. In response to AngII infusion, tyrosine phosphorylation of GIT1 was increased by ~2-fold in the aorta.

Structure-Function Analysis of GIT1 in HEK 293 Cells—To determine the mechanism by which GIT1 regulates receptor-mediated activation of PLCγ, we established a model system using 293 cells, since VSMC are difficult to transfect efficiently with plasmid cDNAs. When transfected with the AngII type 1 receptor (AT1R) cDNA, up to 90% of 293 cells express the AT1R (42) (data not shown). Importantly, in AT1R-expressing 293 cells, AngII (like EGF) stimulates tyrosine phosphorylation of many proteins. To study GIT1 regulation in 293 cells, we first cloned and sequenced the homologous mouse GIT1 cDNA (mGIT1) (Fig. 1A). Comparison of the human, rat, and mouse GIT1 sequences showed that mGIT1 had 89% homology to human GIT1 and 85% homology to rat GIT1. We transfected wild type Xpress-tagged GIT1 cDNA (Xpress-GIT1(wt)) into 293 cells and confirmed that PLCγ bound to GIT1 under basal conditions (Fig. 3A). To verify that both PLCγ and GIT1 were tyrosine-phosphorylated by AngII in 293 cells, we cotransfected Xpress GIT1(wt) and HA-tagged AT1R at a ratio of 3:1. After allowing protein expression for 24 h, the cells were serum-deprived for 16 h and then stimulated with 200 nM AngII for 1 min. In response to AngII, GIT1 and PLCγ were tyrosine-phosphorylated in 293 cells to a similar extent as in VSMC (Fig. 3B). EGF-mediated GIT1 and PLCγ phosphorylation were used as positive controls, since 293 cells express high levels of endogenous EGFR. EGF (10 ng/ml for 5 min) stimulated tyrosine phosphorylation of PLCγ and Xpress-GIT1(wt) in 293 cells to a similar extent as AngII (Fig. 3, C and D, left two lanes). To prove that GIT1 and PLCγ phosphorylation were c-Src-dependent in 293 cells, we repeated the EGF experiment in the presence of a Src kinase inhibitor, 10 μM 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). Activation of PLCγ was assessed by measuring phosphorylation of Tyr783, which has been shown to correlate with PLCγ activity (48).

To assess the effect of GIT1 depletion on PLCγ function, we measured calcium mobilization in VSMC using Fura-2. Cells were stimulated for 1 min with AngII, which rapidly increased intracellular calcium as previously reported (42). Peak intracellular calcium concentration in response to AngII was 223 ± 40 nM, whereas in cells transfected with antisense GIT1 oligonucleotides, peak calcium was 130 ± 73 nM. The percentage increase in calcium concentration stimulated by AngII was significantly different in antisense GIT1 oligonucleotide-transfected cells compared with sense and scrambled GIT1 oligonucleotide-transfected cells (83% inhibition, n = 3). Thus, GIT1 is required for AngII-stimulated calcium mobilization.

GIT1 Plays a Key Role in Stimulation of PLCγ Tyrosine Phosphorylation in VSMC—PLCγ can be activated by several stimuli in VSMC including AngII, thrombin, PDGF, and EGF (43, 44). We initially identified GIT1 as a 97-kDa protein bound to PLCγ by analysis of VSMC after AngII stimulation. However, a more general role for GIT1 in G protein-coupled receptors and tyrosine kinase-coupled receptors has been proposed (34, 45). As shown in Fig. 2A, AngII, thrombin, EGF, and PDGF all stimulated tyrosine phosphorylation of GIT1 in VSMC (from 1.5- to 2.5-fold). We do not believe that AngII-mediated GIT1 phosphorylation was due to transactivation of the PDGF and EGF receptors (46, 47), because AngII stimulated phosphorylation of GIT1 and PLCγ required for GIT1 interaction. Full-length PLCγ contains two SH2 domains (amino-terminal (SH2a) and carboxyl-terminal (SH2c)) and a single SH3 domain (25). Using 293 cells transfected with Xpress-GIT1(wt) and a FLAG-tagged construct of PLCγ that contained only the Src homology domains (PLCγ-SH2a-SH2c-SH3), we performed co-precipitation (Fig. 4A). Immunoprecipitation with anti-FLAG antibody precipi-
FIG. 4. Domains of PLCγ and GIT1 required for interaction. A, in vivo interactions between PLCγ and GIT1. Xpress-GIT1 was co-transfected into 293 cells with vector alone (p-Tag-2c) or FLAG-PLC-γ (containing SH2n-SH2c-SH3). GIT1 was immunoprecipitated with FLAG antibody. The immunoprecipitates were then immunoblotted for the presence of GIT1 using Xpress antibody (top panel), and for PLCγ using FLAG antibody (second panel). Expression of the constructs is shown in the bottom two panels. TCL, total cell lysate. B, in vitro binding assays between Xpress-GIT1(wt) and PLCγ GST fusion proteins containing different variants of the two SH2 domains and the SH3 domain of PLCγ. The top panel is a representative immunoblot, and the bottom panel is quantitative analysis of five independent experiments normalized to PLCγ SH2n-SH2c-SH3 binding of 100%.

FIG. 3. AngII stimulates GIT1 and PLCγ phosphorylation in 293 cells. A, 293 cells were transfected with Xpress-GIT1(wt), and PLCγ was immunoprecipitated. GIT1 was detected with Xpress antibody (upper panel), and PLCγ was detected with PLCγ antibody (lower panel), demonstrating binding under control conditions. B, 293 cells were transfected with Xpress-GIT1(wt) and HA-AT1R and stimulated for 5 min with 200 nM AngII, and cell lysates were immunoprecipitated (IP) with PLCγ antibody. Immunoblotting (IB) was performed using the indicated antibodies. Tyrosine phosphorylation of both GIT1 and PLCγ by AngII was similar to results in VSMC (Fig. 2). C, 293 cells were transfected with PLCγ. Cells were stimulated with 10 ng/ml EGF after treatment for 30 min with 10 μM protein phosphatase 2 or vehicle (Me2SO; DMSO). Cell lysates were immunoprecipitated with PLCγ antibody. Immunoblotting was performed using the indicated antibodies. D, 293 cells were transfected with Xpress-GIT1(wt). Using the same experimental protocol as in C, cell lysates were immunoprecipitated with GIT1 antibody. Immunoblotting was performed using the indicated antibodies. E, cells were stimulated with 10 ng/ml EGF for the indicated times after treatment for 30 min with 10 μM protein phosphatase 2 or vehicle (Me2SO). Cell lysates were immunoprecipitated with EGFR antibody. Immunoblotting was performed using the indicated antibodies (note that pY-EGFR (845) is specific for autophosphorylation).
...immobilized on glutathione-conjugated beads and incubated with cell lysates from 293 cells transfected with PLCγ 1. Lysates were immunoprecipitated with GST antibody and then immunoblotted for the presence of GIT1(del-SHD) using anti-FLAG antibodies (top panel). The left lane shows total cell lysate to confirm protein expression as detected with anti-PLCγ antibody. GST pull down shows an essential domain in aa 250–420. GST (aa 1–250) and GST-GIT1 (aa 250–420) were immobilized on glutathione-conjugated beads and incubated with cell lysates from 293 cells transfected with PLCγ. Beads were washed extensively and then immunoblotted for PLCγ(top panel). Lysates were immunoprecipitated with anti-GST antibodies to confirm equal loading (bottom panel). D, GIT1-CC2 is required for binding to PLCγ. PLCγ was co-transfected with Xpress-GIT1(del-CC2) into 293 cells. Lysates were immunoprecipitated with PLCγ antibody and then immunoblotted for the presence of GIT1(del-CC2) using Xpress antibodies (top panel). Equal expression of proteins was confirmed by blotting total cell lysates with anti-PLCγ or anti-Xpress. To quantify results, the gels were scanned, and the densitometry of GIT1(wt) binding was arbitrarily set to 1.0. Results shown are representative of three experiments.

**Table I**

| Construct | PLC binding |
|-----------|-------------|
| GIT1(wt)  | Yes         |
| GIT1 (aa 1–420) | Yes       |
| GIT1 (aa 1–250) | No        |
| GIT1(aa 250–420) | Yes       |
| GIT1(del-SHD) (aa 250–420) | No       |
| GIT1(del-CC2) (aa 423–471) | Decreased |

Analysis of GIT1 domains required for PLC interaction

Fig. 5. Analysis of GIT1 domains required for PLC binding. A, schema for FLAG-GIT1 and GST-GIT1 deletion constructs. GAP, ARF-GAP; ANK, ankyrin; CC, coiled-coil; SHD, Spa2 homology domain. B, GIT1-SHD is required for binding to PLCγ. PLCγ was co-transfected with FLAG-GIT1(del-SHD) into 293 cells. Lysates were immunoprecipitated with PLCγ antibody and then immunoblotted for the presence of GIT1(del-SHD) using anti-FLAG antibodies (top panel). The left lane shows total cell lysate to confirm protein expression as detected with PITC-gamma or anti-FLAG. C, GST pull down shows an essential domain in aa 250–420. GST (aa 1–250) and GST-GIT1 (aa 250–420) were immobilized on glutathione-conjugated beads and incubated with cell lysates from 293 cells transfected with PLCγ. Beads were washed extensively and then immunoblotted for PLC-gamma (top panel). Lysates were immunoprecipitated with anti-GST antibodies to confirm equal loading (bottom panel). D, GIT1-CC2 is required for binding to PLCγ. PLCγ was co-transfected with Xpress-GIT1(del-CC2) into 293 cells. Lysates were immunoprecipitated with PLCγ antibody and then immunoblotted for the presence of GIT1(del-CC2) using Xpress antibodies (top panel). Equal expression of proteins was confirmed by blotting total cell lysates with anti-PLCγ or anti-Xpress. To quantify results, the gels were scanned, and the densitometry of GIT1(wt) binding was arbitrarily set to 1.0. Results shown are representative of three experiments.

Identification of the GIT1 Binding Domains Required for Interaction with PLCγ—GIT1 is composed of a zinc finger-containing ARF GAP domain (GAP), ankyrin repeat region (ANK), two carboxyl paxillin-binding subdomains, a Spa2 homology domain (SHD), and three putative coiled coil (CC) domains (Fig. 5A). The ARF-GAP domain has been shown to be functionally important for endocytosis (45). Interestingly, only GIT1 (amino acids 250–396) and yeast Spa2 family members contain SHD motifs (1). The CC regions include CC1 (residues 253–274), which overlaps with the SHD domain, CC2 (residues 424–474), and CC3 (residues 649–669) (53). The CC2 region is important for interactions with PAK (32), the CC3 (including 646–770) is essential for interactions with paxillin (53), and the SHD region overlaps with binding sites defined for focal adhesion kinase and PIX (53). To identify the regions in GIT1 that interact with PLCγ, we made deletion mutations of these putative protein interacting domains as described (Fig. 5A).

Because the SHD domain and CC1 overlap, we first investigated the requirement for this domain in GIT1-PLCγ binding. As shown in Fig. 5B, FLAG-GIT1(del-SHD), which lacks aa 250–420, was unable to bind PLCγ in transfected 293 cells. In contrast, GIT1 (aa 1–420) and GIT1 (aa 250–770) were able to bind PLCγ (Table I). These results suggested that critical binding motifs were present in aa 250–420. To provide further evidence for binding via the SHD domain, we compared the ability of GST-GIT1 (aa 1–250) and GST-GIT1 (aa 250–420) to precipitate PLCγ in a pull-down assay. As expected, PLCγ was pulled down by GST-GIT1 (aa 250–420) but not by GST-GIT1 (aa 1–250) (Fig. 5C). To investigate the role of the CC2 domain we transfected cells with GIT1(del-CC2) and GIT1(wt). In con-
comparison with GIT1(wt), precipitation of GIT1(del-CC2) by anti-PLCγ antibody was significantly decreased (percentage decrease in GIT1(del-CC2) binding was 69±10%, n = 3; Fig. 5D, Table I). These results indicate that the SHD and CC2 domains play important roles in GIT1 interactions with PLCγ.

To determine the functional significance of GIT1 interacting with PLCγ, we studied EGF and AngII activation of PLCγ (phosphorylation of Tyr783) in 293 cells transfected with vector control, Flag-GIT1 (wt), or Flag-GIT1(del-SHD). Several studies have shown essential roles for Src-dependent tyrosine phosphorylation of PLCγ in mediating activation by EGF (50) and AngII (12). In cells transfected with vector control, 10 ng/ml EGF activated PLCγ at both 2 and 10 min as measured by Tyr(P)783 (Fig. 6A). Maximal activation (1.7 ± 0.7-fold increase) occurred at 10 min (Fig. 6D). In cells transfected with GIT1(wt), the time course was similar to control (Fig. 6B), although the magnitude of Tyr(P)783 was significantly greater at 5.6 ± 0.6-fold at 10 min (Fig. 6D). In contrast, in cells transfected with GIT1(del-SHD), Tyr(P)783 at 10 min was significantly less than GIT1(wt) (3.1 ± 0.3-fold) (Fig. 6C). In response to EGF at 2 min, the increase in Tyr(P)783 was similar in GIT1(wt) and GIT1(del-SHD), suggesting that phosphorylation of PLCγ may involve several mechanisms. To prove that phosphorylation of PLCγ Y783 was functionally important, we measured EGF-stimulated Ins(1,4,5)P3 formation (Fig. 6E). Results similar to those observed for Tyr(P)783 were observed. Specifically, transfection of GIT1(wt) or GIT1(del-SHD) had no effect on basal Ins(1,4,5)P3 levels (Fig. 6E). In response to EGF, there was a 3.2-fold increase in Ins(1,4,5)P3 formation in cells transfected with GIT1(wt). This was significantly greater than the 1.7- and 1.9-fold increases observed in cells transfected with vector and GIT1(del-SHD), respectively (n = 3, p < 0.05; Fig. 6E). These data indicate that GIT1 binding to PLCγ (via the SHD domain) is required for phosphorylation of Tyr783 and stimulation of PLCγ activity.

To evaluate whether the requirement for GIT1 binding to PLCγ via the SHD domain held true for AngII, we repeated the experiments in 293 cells cotransfected with the AT1R, PLCγ, and GIT1 constructs. As shown in Fig. 7A, AngII stimulated Tyr(P)783 maximally at 5 min (2.5-fold increase) in cells transfected with vector control. Transfection of GIT1(wt) did not alter the time course but significantly increased Tyr(P)783 compared with control (5.6-fold increase). In contrast, GIT1(del-SHD) was not significantly different from control at 0 min.
Interactions of GIT1 with PLCγ and c-Src—An unanswered question is how AngII binding to the AT1R leads to tyrosine phosphorylation of PLCγ and GIT1. To determine whether there were direct interactions of the AT1R and c-Src with either GIT1 or PLCγ, we performed co-immunoprecipitation experiments. Neither PLCγ nor GIT1 directly associated with the AT1R based on this analysis, since immunoprecipitation of the AT1R did not co-precipitate PLCγ or GIT1, nor was the AT1R present in PLCγ and GIT1 immunoprecipitates from VSMC (data not shown). Similarly, c-Src was not present in these immunoprecipitates. To determine whether GIT1 interacted with these proteins when overexpressed, we cotransfected GIT1(wt) and the AT1R into 293 cells. GIT1(wt) did not directly associate with the AT1R or c-Src in 293 cells (data not shown). Thus, the interactions between these proteins are transient and/or other proteins are involved.

**DISCUSSION**

The major findings of this study are the identification of GIT1 as a novel mediator for activation of PLCγ that appears to integrate signal transduction from both GPCRs and TKRs. Binding of GIT1 to PLCγ requires the Spa2 homology domain (aa 250–470), a domain whose function has only been defined in yeast. In addition, GIT1 appears to be a substrate for c-Src phosphorylation in response to activation of both GPCRs and TKRs and may explain, in part, the ability of AngII to activate PLCγ. Several independent experimental approaches support an essential role for GIT1 in PLCγ function. Knockdown of GIT1 expression significantly decreased PLCγ activation. Transfection of a GIT1 mutant that does not bind PLCγ, termed GIT1(del-SHD), prevented PLCγ activation as shown by decreased PLCγ Y783 phosphorylation and Ins(1,4,5)P3 formation. Treatment of cells with the c-Src inhibitor protein phosphatase 2 blocked AngII- and EGF-mediated phosphorylation of both GIT1 and PLCγ, indicating the importance of c-Src as a common mediator. These data support previous studies documenting an important role for Src-dependent tyrosine phosphorylation in PLCγ activation and calcium mobilization in several cell types (50, 54, 55). In addition, results from our laboratory in Src−/− cells and with c-Src inhibitors showed a correlation between decreased GIT1 tyrosine phosphorylation and activation of PLCγ (23, 26).

The central portion of GIT1 (aa 250–470) encompassing the SHD, CC1, and CC2 domains is required for regulation of PLCγ. PLCγ activation requires PLCγ interaction with the SHD and CC1 (and to a lesser extent CC3) domains of GIT1 and binding of GIT1 to the SH2-SH3 domains of PLCγ. Future studies will be required to define the specific mechanisms that mediate activation of PLCγ when bound to GIT1, since binding is constitutive. It is likely that c-Src-mediated phosphorylation of specific tyrosine residues in regions of GIT1 will be important based on our finding that tyrosine phosphorylation was required for agonist-mediated Ins(1,4,5)P3 formation. Based on the present study, we propose a model to explain the role of GIT1 in activation of PLCγ by AngII and EGF (Fig. 8). In serum-starved cells, GIT1 exists in a preassembled complex with PLCγ. Upon agonist binding (for both GPCRs and TKRs), c-Src is activated and phosphorylates critical tyrosine residues in GIT1. We propose that a conformational change in GIT1 now recruits additional signaling molecules (e.g., focal adhesion kinase) and/or enables c-Src to phosphorylate additional tyrosine residues (e.g., PLCγ Tyr783) that lead to activation of PLCγ. This pathway is separate from the transactivation pathway for GPCR-mediated tyrosine phosphorylation of the EGFR by generation of heparin-binding EGF.

The newly defined role of GIT1 in PLCγ activation and Ins(1,4,5)P3 formation described here complements the known roles of GIT family members. Previous studies indicate important roles for GIT proteins in receptor endocytosis (27, 34, 45) and cell motility (53, 56). GIT1 (also termed Cat-1 for Cool cloned out of library)-associated tyrosine-phosphorylated protein) was originally identified in a yeast two-hybrid screening for proteins that bind G protein-coupled receptor kinase 2 (27). Initial results demonstrated that GIT1 functions as a GAP for the ARF family of proteins, and GIT1 was shown to regulate both GPCR and TKR receptor internalization. GIT proteins are expressed ubiquitously in human tissues (27, 31) so that GIT family members are likely to regulate many membrane receptors. Work from Zhao et al. (53) and from Turner and colleagues (32, 56) supports an important role for GIT family members in focal adhesions and cell motility. Specifically, these authors
proposes that upon cell activation, Cdc42 recruitment of PAK and PXK drives the association of GIT1 with focal adhesions. This favors dissociation of paxillin from focal adhesions that become destabilized and promotes motility by decreasing cell adherence. The association of GIT proteins with PAK, PXK, and PXJ suggests a functional role for GIT proteins in regulation of the cytoskeleton (29, 31, 59). Because of the important role of calcium in cell motility and focal adhesion function (e.g., Pyk2 is a calcium-dependent focal adhesion kinase), the present study suggests that GIT1 may be involved in local regulation of calcium homeostasis. Future work will be required to define the possible roles of known GIT1-interacting proteins in regulation of PLCγ, especially at specific intracellular sites such as focal adhesions.

To date only one PLC scaffold has been described, the Dro sophila photoreceptor PDZ scaffold protein INAD, which acts with PLC-γ (57). In this system, the association between PLC and INAD is important to terminate the light response via PLC functioning as a GAP. PLC and INAD is important to terminate the light response via PLC functioning as a GAP. PLC and INAD is important to terminate the light response via PLC functioning as a GAP. PLC and INAD is important to terminate the light response via PLC functioning as a GAP. PLC and INAD is important to terminate the light response via PLC functioning as a GAP.