GIT1 Functions as a Scaffold for MEK1–Extracellular Signal-Regulated Kinase 1 and 2 Activation by Angiotensin II and Epidermal Growth Factor

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Activation of the mitogen-activated protein kinase pathway represented by extracellular signal-regulated kinases (ERK1/2) and activation of the upstream kinase (MEK1) are critical events for growth factor signal transduction. c-Src has been proposed as a common mediator for these signals in response to 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 associates with MEK1 in vascular smooth-muscle cells and human embryonic kidney 293 cells. GIT1 binding via coiled-coil domains and a Spa2 homology domain is required for sustained activation of MEK1-ERK1/2 after stimulation with angiotensin II and epidermal growth factor. We propose that GIT1 serves as a scaffold protein to facilitate c-Src-dependent activation of MEK1-ERK1/2 in response to both GPCRs and TKRs.

Several signal transduction events induced by angiotensin II (AngII) binding to the AngII type 1 receptor (AT1R) resemble those stimulated by epidermal growth factor (EGF) and platelet-derived growth factor binding to their receptors (33, 42). AngII stimulates cell proliferation and cell hypertrophy of many cell types, including vascular smooth-muscle cells (VSMC), cardiac fibroblasts, mesangial cells, and macrophages (5, 15, 39). A key role for tyrosine kinases (such as c-Src) in AngII- and EGF-mediated increases in intracellular calcium and activation of mitogen-activated protein (MAP) kinases has been established (4). 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 (32). Rapid tyrosine phosphorylation is likely mediated by nonreceptor tyrosine kinases, including Src family tyrosine kinases JAK2 and PYK2 (1, 6, 13, 19, 24, 31, 35). A key role for Src in activation of extracellular signal-regulated kinases (ERK1/2) was shown by nearly complete inhibition of AngII-stimulated ERK1/2 activation in Src^{-/-} cells as well as inhibition by protein phosphatase 1 (PP1) and genistein (18). ERK1/2 activation by AngII is responsible for the induction of early-growth-response genes, including *c-fos* and *c-jun* (33, 42). Other important c-Src substrates in VSMC include phospholipase C- γ , which becomes tyrosine phosphorylated rapidly in response to AngII (18, 38). c-Src also plays a role in AngII signaling by mediating activation of focal adhesion kinase (FAK) (37) and transactivation of the EGF receptor (EGFR) and platelet-derived growth factor receptor (16, 47).

The EGFR is a ubiquitously expressed transmembrane receptor tyrosine kinase. Activation of ERK1/2 by the EGFR

involves sequential assembly of a signaling complex via autophosphorylation of tyrosines and SH2-mediated interactions with Grb2 and Shc. These proteins then recruit the guanine nucleotide exchange protein son of sevenless (Sos). Sos catalyzes GDP release and GTP binding to Ras and activates ERK1/2. c-Src is also recruited to the EGFR, and this interaction is required for many EGFR-mediated cellular functions, including proliferation, migration, survival, and EGFR endocytosis (3, 22). Specifically, c-Src promotes EGF-induced PI3 kinase activation and DNA synthesis via tyrosine phosphorylation of Grb2 (21).

To understand the role of Src kinases in AngII-mediated signal transduction, we have focused on ERK1/2 activation as a key rapid event. Here, we demonstrate that GIT1, the G protein-coupled receptor kinase-interacting protein (29), is a key regulator of AngII- and EGF-mediated ERK1/2 activation in VSMC and human embryonic kidney (HEK) 293 cells. Recent reports have described at least two GIT family members with numerous tissue-specific alternatively spliced isoforms: GIT1 (also termed Cat-1 Cool [for “cloned out of library”]-associated tyrosine-phosphorylated protein) (2) and GIT2 (the product of the KIAA0148 gene) (30, 45), also termed paxillin kinase linker protein (43). All GIT family members share a structure composed of an amino-terminal zinc finger-like motif, an ADP ribosylation factor (ARF) GTPase-activating protein (GAP) domain, three ankyrin repeats, and a conserved carboxyl-terminal region that interacts with paxillin. GIT1 and GIT2 are active as GAPs for ARF1 and ARF6 (45) and bind GRK2. GIT1 affects the function of receptors (both G protein-coupled receptors [GPCRs] and tyrosine kinase-coupled receptors [TKRs]) that are internalized through the clathrin-coated pit pathway in a β -arrestin- and dynamin-sensitive manner (10). All GIT family members appear to bind a complex that includes the guanine nucleotide exchange factor PIX and the p21 GTPase-activated kinase PAK (2, 30, 43). In the present report, we show that GIT1 links the AT1R and the

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EGFR to ERK1/2 activation by associating with MEK1. Our results demonstrate a novel role for GIT1 as a scaffold for c-Src-dependent signal transduction activated by GPCRs and TKRs.

MATERIALS AND METHODS

Cell culture. VSMC were obtained from rat aorta as described previously (26). HEK 293 cells and VSMC were grown in cultures in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

Plasmid cDNAs. The mGIT1-expressed sequence tag clone (GenBank accession number A1414223) 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 a reverse transcriptase reaction using mouse brain total RNA and specific primers (5'-CTGAGCTGGAGAGC TTAGATGGAG ACC-3' and 5'-GCTCTAGAGGTCCAGGGTGTGGGTA AGGGCAG-3'). Then, full-length mGIT1 [GIT1(wt)] was cloned into the *NorI* and *XbaI* sites of Xpress-tagged pcDNA3.1 vector [resulting in Xpress-GIT1(wt)] and the *EcoRI* and *ApaI* sites of pCMV-Tg2C vector [Flag-GIT1(wt)]. Using PCR, GIT1(1 to 635aa), GIT1(1-420aa), GIT1(420-770aa), GIT1(250-770aa), and GIT1(del-SHD) were cloned into pCMV-Tg2B vector [resulting in Flag-GIT1(1-635aa), Flag-GIT1(1-420aa), Flag-GIT1(420-770aa), Flag-GIT1(250-770aa), and Flag-GIT1(del-SHD)]. GIT1(del-CC2) and GIT1(Y321F) mutants were obtained using QuikChange site-directed mutagenesis (Stratagene). Using PCR, GIT1(1-250aa), GIT1(250-420aa), and MEK1(70-200aa) were cloned into the *BamHI* and *XhoI* sites of pGEX-KG [resulting in GST-GIT1(1-250aa), GST-GIT1(250-420aa), and GST-MEK1(70-200aa)]. The insert sequence and reading frame were confirmed by sequencing. Src(Y527F) and Src(Y416F) and Src(K295R) cDNAs were a generous gift from Jonathan A. Cooper (Univ of Washington).

Transfection with cDNAs, antisense oligonucleotides, and RNA interference. HEK293 cells were transfected by Lipofectamine Plus (GIBCO BRL). VSMC were transfected using FuGENE 6 reagent (Roche Molecular Biochemical). For cotransfections, a ratio of 3:1 was used. After allowing protein expression for 24 h, cells were serum deprived for 24 h and stimulated with agonists. Phosphorothiolated sense (S), scrambled, or antisense (AS) oligonucleotides (VSMC; 5 µg) corresponding to the GIT1 sequence (S-GIT1, 5'-CAACTTCATCTGGGA GCACTC-3'; AS-GIT1, 5'-CTGATGAACCTGACTTGATGG-3') were transfected into VSMC according to the manufacturer's protocols. Three RNA interference (RNAi) constructs (1A, 2A, and 3A) were created using pSHAG (kindly provided by Greg Hannon, Cold Spring Harbor Laboratories). Briefly, oligonucleotides carrying short RNA hairpins targeted to conserved regions of human (NM_014030) and mouse (XM_126291) GIT1 were annealed and cloned into *BseRI*-*BamHI*-cut pSHAG just downstream of the U6 promoter. The sequences of the oligonucleotides used were as follows: for construct 1A, oligonucleotides 1A' (5'-TAGGCGCTGGCGTTGAGCAGCCGAGTGAAGCTTG CGCTGCGGCTGCTTAACGCTAGCGCTTACCGTTT-3') and 1B' (5'-GATCAAAAAACGGAAGCGCTAGCGTTAAGCAGCCGAGCGCAAG CTTCCTACTGCGCTGCTCAACGCCAGCGCCTACG-3'); for construct 2A, oligonucleotides 2A' (5'-GTGACCAAGCTGCTTGGCAGCCTTGGCGAGAA GCTTGTGCTAAGGCTGCTAAGCAGCTGCTTACCATT-3') and 2B' (5'-GATCAAAAAAATGGTAACCAAGCTGCTTACGAGCCTTAGCAA CAAGCTTCTCGCAAGGCTGCCAAGCAGCTGGTACCG-3'); and for construct 3A, oligonucleotides 3A' (5'-CTCCAGGTACTCTGCAGCGTCA CAGCCGAAGCTTGGGCTGTGGCGTTGTAGGAGTACTGGAGCTGTT TTTT-3') and 3B' (5'-GATCAAAAAACAGCTCCAAGTACTCTACAACG CCACAGCCCAAGCTTGGGCTGTGACGCTGCAGGAGTACCTGGAGC G-3').

Yeast two-hybrid screening and yeast mating tests. Using PCR, we amplified the full-length mouse cDNA of GIT1; the fragment was then cloned into pGBKT7 vector, resulting in a GIT1 bait expression construct (pGBKT7-GIT1). The insert sequence and reading frame were confirmed by sequencing. After pGBKT7-GIT1 vector was transformed into AH109 by a lithium acetate-mediated method, AH109/pGBKT7-GIT1 was obtained. After the 11-day mouse embryo cDNA library (Clontech) was sequentially transformed into AH109/pGBKT7-GIT1, 1,200 positive-testing clones were grown on synthetic dropout (SD)/Trp-Leu-Ade-His-. When a colony-lift filter assay designed to detect β-galactosidase activity was used, only 150 positive-testing clones were obtained. Of these clones, 40 specifically interacted with GIT1 in yeast AH109/Y18 mating tests. Sequence analysis and bioinformatics studies indicated that 6 out of 40 GIT1-interacting sequences represented MEK1.

FIG. 1. Genetic complementation in yeast shows specific interaction between MEK1 and GIT1. Complementation analysis was performed as described in Materials and Methods. The positive control was p53 protein interacting with transcription factor TD-1 (pGBKT7-p53+pACT2-MEK1) and with vector alone (pGBKT7+pACT2-MEK1). In contrast, MEK1 interacts with GIT1 (pGBKT7-GIT1+pACT2-MEK1) to the same extent as the positive control.

Immunoprecipitation and immunoblotting. Anti-glutathione *S*-transferase (GST) monoclonal antibody (MAb) and ERK1/2 polyclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-GIT1 and -MEK1 MABs were purchased from BD Transduction Laboratories (Lexington, Ky.). Anti-Flag M2 and antihemagglutinin (anti-HA) MABs were received from Sigma (St. Louis, Mo.). Anti-Xpress MAB was purchased from Invitrogen. Anti-pERK1/2 and anti-pMEK1/2 were obtained from Cell Signaling (Beverly, Mass.). For immunoprecipitations, cells were lysed in radioimmunoprecipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% sodium dodecyl

FIG. 2. GIT1 and MEK1 interact specifically in HEK 293 cells. (A) Cells were transfected with Xpress-GIT1(wt) and HA-MEK1 and immunoprecipitated (IP) with anti-HA antibody. Note that for all figures showing proteins that were expressed from transfected cDNAs and detected on Western blots (IB), the results are indicated on the right side of the panel (IB: HA and IB: Xpress). Blots were probed with anti-Xpress to detect Xpress-GIT1 that coprecipitated with HA-MEK1 but not with control serum (IgG = rabbit IgG). (B) Cells were transfected as described for panel A, and cell lysates were immunoprecipitated with anti-Xpress antibody and probed with anti-HA to detect HA-MEK1 that coprecipitated with GIT1. The blots were re-probed with anti-Xpress to confirm GIT1 expression and with anti-HA to confirm MEK1 expression in total cell lysates (TCL).

FIG. 3. GIT1 and MEK1 associate constitutively in VSMC and HEK 293 cells, and the interaction is not altered by the presence of AngII or EGF. (A) The association of endogenous MEK1 and GIT1 in VSMC was assayed by immunoprecipitation (IP) with anti-MEK1 antibody and probing for GIT1. Although GIT1 coprecipitated with MEK1, there was no change in the association in response to the presence of AngII (200 nM, 2 min) or EGF (10 ng/ml, 5 min). Control immunoprecipitation was performed with rabbit IgG (IgG). Equal amounts of MEK1 were precipitated as shown by probing with anti-MEK1 (bottom panel). (B and C) The time course seen with HEK 293 cells for association between transfected Xpress-GIT1(wt) and HA-MEK1 in response to the presence of EGF (B) and AngII (C) was determined at the indicated times using anti-HA to precipitate MEK1 and anti-Xpress to detect GIT1.

sulfate, 50 mM Tris-HCl, pH 8.0) with inhibitor (0.5 μ g of leupeptin/ml, 1 mM EDTA, 1 μ g of pepstatin A/ml, 0.2 mM phenylmethylsulfonyl fluoride). Analysis of autoradiograms after immunoblotting was performed by scanning densitometry and processed with National Institutes of Health Image software. Statistical analysis was performed using Student's *t* test.

Protein-protein interaction assays. HEK293 cells were cotransfected with Xpress-GIT1(wt) or pcDNA3.1 vector with HA-MEK1 and Lipofectamine Plus. Cells were harvested 24 h after transfection and lysed in radioimmunoprecipitation buffer. After sonication at 50 kHz for 6 s, cell lysates were centrifuged (maximum speed, 10 min, 4°C). Approximately 500 μ g of precleared lysates were immunoprecipitated using 2 μ g of anti-HA and rabbit immunoglobulin G (IgG). They were then separated and probed with anti-Xpress and anti-HA antibodies and then with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences UK Limited) and visualized using an enhanced chemiluminescence technique. HEK293 cells were cotransfected using HA-MEK1 or pcDNA3.1 with Xpress-GIT1, immunoprecipitated using anti-Xpress, and probed with anti-HA antibody and anti-Xpress antibodies.

RESULTS

Yeast two-hybrid screening for GIT1-interacting proteins: identification of MEK1. To define Src kinase substrates that mediate GPCR signal transduction, we characterized proteins rapidly tyrosine phosphorylated in an Src-dependent manner. Our strategy was to use both pharmacological inhibition and Src^{-/-} cells to immunoprecipitate proteins from VSMC that associated with phospholipase C- γ and to identify proteins

dependent on Src. This analysis yielded a 97-kDa protein (p97) that was tyrosine phosphorylated in response to the presence of AngII (38). p97 was excised from a silver-stained sodium dodecyl sulfate-polyacrylamide gel, digested with trypsin, and subjected to mass spectrometry and microsequence analysis (12). Comparison with the SWISS-PROT protein sequence database entries yielded a complete match with a GIT1 previously identified by Premont et al. (29).

To identify GIT1-interacting proteins that may be involved in c-Src-mediated signal transduction, we performed a yeast two-hybrid screen. A full-length mouse GIT1 GAL4 binding domain construct was cotransformed into the yeast strain AH109 with a GAL4 activation domain fusion library of mouse embryo cDNA. From 1,200 clones screened, 150 positive-testing clones were identified; 40 were confirmed by genetic complementation. Among these 40 clones, 33 encoded proteins with annotated functions and 7 were expressed sequence tags. There were six clones identical to MEK1, which has been identified as an upstream activator of ERK1/2. As shown in Fig. 1, none of the MEK1 clones (pACT2-MEK1) interacted with the GAL4 DNA binding domain alone (pGBKT7) or with a control bait protein (pGBKT7-53). In contrast, GIT1 (pGBKT7-GIT1) interacted with MEK1(pACT2-MEK1) to

FIG. 4. Diagram of GIT1 deletion mutants and GIT1-GST fusion proteins. Flag-GIT1 constructs and GST-GIT1 fusion proteins were prepared as described in Materials and Methods.

the same extent as the positive controls (pGBKT7-53 and pTD1-1).

GIT1-MEK1 binding is physiologically important. The following experiments were performed to show that the interaction between MEK1 and GIT1 is specific and has functional consequences. We used HEK 293 cells for the experiments described below because of their high level of transfection efficiency, abundance of Src-dependent signal events, and relatively low-level expression of endogenous GIT1. We cotransfected HEK 293 cells with Xpress-GIT1 and HA-MEK1 expression plasmids. Immunoprecipitation using either anti-HA (Fig. 2A) or anti-Xpress (Fig. 2B) antibodies demonstrated that MEK1 and GIT1 coprecipitated. Comparable amounts of control rabbit IgG failed to precipitate GIT1 or MEK1. We next investigated the interaction of GIT1 and MEK1 in VSMC, which express high levels of GIT1. In cells serum starved for 24 h, GIT1 associated with MEK1, as shown by immunoprecipitation with anti-MEK1 antibody (Fig. 3A) but not by immunoprecipitation with control rabbit IgG. To determine whether agonists altered GIT1-MEK1 interactions, cells were stimulated with 200 nM AngII or 10 ng of EGF/ml for times associated with peak MEK1 activation (2 or 5 min, respectively). The interaction between endogenous GIT1 and MEK1 was not affected by the presence of either EGF or AngII (Fig. 3A). We also tested the association between Xpress-GIT1 and HA-MEK1 in HEK 293 cells stimulated with EGF (Fig. 3B) and with AngII after cotransfection with the AT1R (HA-AT1R; Fig. 3C). The association between GIT1 and MEK1, as determined by immunoprecipitation with anti-HA, was not changed by EGF or AngII stimulation.

Domains of GIT1 that mediate interaction with MEK1. GIT1 is composed of a zinc finger-containing ARF GAP domain, an ankyrin repeat region, two carboxyl paxillin-binding subdomains, a Spa2 homology domain (SHD), and three putative coiled-coil (CC) domains (Fig. 4). The ARF-GAP domain has been shown to be functionally important for endocytosis (9). Interestingly, only GIT1(250-396aa) and yeast Spa2

family members contain SHD motifs (40). The CC regions include CC1 (residues 253 to 274), which overlaps with the SHD domain, CC2 (residues 424 to 474), and CC3 (residues 649 to 669) (53). The CC2 region is important for interactions with PAK (43), the CC3 region (including residues 646 to 770) is essential for interactions with paxillin (53), and the SHD region overlaps with binding sites defined for FAK and PIX (53). Importantly, the yeast SHD has recently been shown to bind to Mkk1p, Mkk2p, and Ste7p, suggesting that this would be a likely domain for interaction with MEK1 (44).

To define the domains responsible for GIT1-MEK1 interactions we transfected HEK 293 cells with the HA-MEK1 and GIT1 deletion mutants described for Fig. 4. Immunoprecipitation of MEK1 with anti-HA antibody coprecipitated GIT1(250 to 770aa), GIT1(1 to 635aa), and GIT1(1 to 420aa) but not GIT1(420 to 770aa) or GIT1(del-SHD), which is missing amino acids (aa) 250 to 420 (Fig. 5A, Table 1). The fact that GIT1(250 to 770aa) also coprecipitated with MEK1 suggested that essential amino acids were present in a binding domain that encompassed aa 250 to 440. To prove the role of these amino acids and demonstrate a direct interaction, we used a GST pulldown assay. As shown in Fig. 5B, MEK1 was pulled down by GST-GIT1(250 to 420aa) but not by GST(1 to 250aa) or GST alone. To define the interactions of this region in greater detail, we specifically deleted the CC2 domain (aa 421 to 471). Deleting the CC2 domain [GIT1(del-CC2)] significantly decreased MEK1 binding (Fig. 5C), although not to the same extent as that observed for GIT1(del-SHD). Taken together, these data suggest that the SHD domain and (to a lesser extent) the CC2 domain are essential for the interaction of GIT1 with MEK1 (Table 1).

Functional effects of GIT1-MEK1 interactions. Having established that GIT1 and MEK1 associate under basal conditions, we tested the functional consequences of GIT1 interactions with MEK1 on the MEK1-ERK1/2 pathway. We transfected HEK293 cells with GIT1 or pcDNA3 and stimulated them with 10 ng of EGF/ml for 2 to 30 min. In cells

FIG. 5. Domains of GIT1 required for interaction with MEK1. The results of *in vivo* interactions between MEK1 and GIT1 are shown. The Flag-GIT1 constructs described for Fig. 4 were cotransfected into HEK 293 cells with HA-MEK1. MEK1 was immunoprecipitated (IP) with anti-HA. The immunoprecipitates were then immunoblotted (IB) using anti-Flag antibodies for detection of the presence of GIT1 (top panel). Note that the lanes shown were individually selected to show interactions of GIT1 constructs of the appropriate molecular weights. All constructs bound to MEK1 except for GIT1(420-770aa) and GIT1(del-SHD). Probing with anti-HA (middle panel) and anti-Flag (bottom panel) showed equal levels of expression of MEK1 and GIT1, respectively. (B) GIT1(250-420aa), but not GIT1(1-250aa), coprecipitates MEK1. GST, GST-GIT1(1-250aa), and GST-GIT1(250-420aa) were immobilized on glutathione-conjugated beads and incubated with total cell lysate from HA-MEK1-transfected HEK 293 cells. Beads were washed extensively and then immunoblotted for HA-MEK1 (top panel). GST, GST-GIT1(1-250aa), and GST-GIT1(250-420aa) were immunoblotted with anti-GST antibodies to confirm equal loading (bottom panel). (C) HA-MEK1 was cotransfected with Xpress-GIT1(wt) and Xpress-GIT1(del-CC2) into HEK 293 cells. Lysates were immunoprecipitated with anti-HA and then immunoblotted using anti-Xpress antibodies to detect the presence of GIT1 (top panel). To confirm equal protein immunoprecipitation, the blot was reprobed with anti-HA (middle panel). To confirm equal protein expression, total cell lysates (TCL) were blotted with anti-HA (bottom panel).

transfected with pcDNA3, EGF rapidly induced ERK1/2 phosphorylation, with a peak at 2 to 5 min and a return to the baseline at 10 min (Fig. 6A). Overexpression of GIT1(wt) increased the duration of ERK1/2 activation, with persistent

phosphorylation at 10 min and a return to the baseline at 30 min. To prove that GIT1 regulated ERK1/2 phosphorylation, we sought additional evidence supporting the functional significance of their association. We transfected HEK 293 cells with vector alone (pcDNA3), GIT1(wt), GIT1(del-SHD), and GIT1(del-CC2) and then stimulated them with EGF and measured ERK1/2 activity by Western blot analysis with phosphospecific ERK1/2 (pERK1/2) antibody (Fig. 6B). In cells transfected with vector alone (pcDNA3), EGF stimulated pERK1/2 maximally (7.0-fold) at 5 min. In cells transfected with GIT1(wt), there was no significant difference in EGF stimulation of pERK1/2 at 5 min compared to the results seen with vector (Fig. 6B) (GIT1[wt]). In cells transfected with GIT1(wt), however, there was a dramatic increase in EGF-stimulated pERK1/2 levels at 10 min (3.5-fold increase versus

TABLE 1. Analysis of GIT1 domains required for MEK1 interaction

Construct	MEK1 binding
GIT1 (wt).....	Yes
GIT1 (1-635).....	Yes
GIT1 (1-420).....	Yes
GIT1 (250-770).....	Yes
GIT1 (420-770).....	No
GIT1 (del-SHD)	No
GIT1 (del-CC2)	Decreased

FIG. 6. GIT1(wt) enhances AngII- and EGF-stimulated ERK1/2 activity specifically. (A) HEK 293 cells were transfected with Xpress-GIT1(wt) or pcDNA3 for 24 h and then serum starved for 16 h and stimulated with 10 ng of EGF/ml for the indicated times. ERK1/2 phosphorylation was measured with phosphospecific ERK1/2 antibody. IB, immunoblotting. (B) HEK 293 cells were transfected with the indicated GIT1 cDNAs or vector alone (pcDNA3) for 24 h and then serum starved for 16 h and stimulated with 10 ng of EGF/ml for the indicated times. ERK1/2

FIG. 7. Effect of GIT1 RNAi on EGF signaling and of antisense GIT1 oligonucleotides on AngII signaling. (A) HEK 293 cells were transfected with Xpress-GIT1 and the indicated concentrations of three different RNAi constructs (or GFP as the control) as described in Methods and Materials. GIT1(wt) expression was measured using anti-Xpress antibody (top panel). ERK1/2 activity was measured with phosphospecific ERK1/2 antibody (middle panel), and protein equal loading was confirmed by ERK1/2 antibody (bottom panel). IB, immunoblotting. (B) HeLa cells were transfected with 4 µg of either GIT1 RNAi-3A or GFP RNAi for 36 h and starved for 16 h. Cells were treated with 10 ng of EGF/ml as indicated. ERK1/2 activity was measured with phosphospecific ERK1/2 antibody (top panel), and equal levels of protein loading were confirmed using ERK1/2 antibody (middle panel). Endogenous GIT1 was probed with GIT1 antibody (bottom panel). (C) The relative increase of ERK1/2 phosphorylation compared to control results (Fig. 7B) was determined by performing quantitative densitometry (Θ , $P < 0.01$ [mean $\pm$ SE; $n = 3$]). (D) The effect of antisense GIT1 oligonucleotides on AngII-stimulated (200 nM, 2 min) ERK1/2 activity in VSMC was measured with phosphospecific ERK1/2 antibody (p-ERK1/2). ERK2 and endogenous GIT1 were detected by reprobing with the indicated antibodies.

the results seen with vector; $n = 5$, $P = 0.01$) (Fig. 6B and 6C) consistent with the data in Fig. 6A. Consistent with the data in Fig. 5 for binding of these GIT1 mutants to MEK1, the results seen with GIT1(del-SHD) and GIT1(del-CC2) did not differ significantly from those seen with the vector at any time point.

To prove that the effects seen with GIT1(wt) were related to MEK1 binding and activity, we repeated the experiment and assayed for MEK1/2 activation with a phosphospecific MEK1/2 (pMEK1/2) antibody (Fig. 6B, right panel). In cells transfected with the control vector, EGF stimulated MEK1 as a rate faster than that seen with ERK1/2 (onset at 2 min). Consistent with the ERK1/2 results, GIT1(wt) increased pMEK1/2 levels to a greater extent than the vector (especially at 10 min [3.0-fold increase versus vector results]). In contrast to GIT1(wt) results, GIT1(del-SHD) and GIT1(del-CC2) results did not differ from vector results (Fig. 6B and 6D). To evaluate the effect of GIT1 on AngII signaling, we cotransfected HEK 293 cells

with the AT1R as well as GIT1 constructs (Fig. 6E). In similarity to the results seen with EGF, AngII-stimulated ERK1/2 phosphorylation was significantly increased in HEK 293 cells transfected with GIT1(wt) compared to that seen with vector alone, especially at 10 min (twofold greater increase than that seen with control) (Fig. 6E, F, and G). Consistent with the ERK1/2 results, GIT1(wt) increased pMEK1/2 levels to a greater extent than the vector (especially at 10 min [2.1-fold increase versus vector results]). The effect of GIT1 was specific for ERK1/2, as there was no change in activation of p38 in cells transfected with GIT1(wt) compared to the results seen with the vector alone (Fig. 6H). There was no difference in the expression of MEK1 or ERK1/2 under any conditions studied.

GIT1 knockdown inhibits ERK1/2 activation. To provide evidence for the function of GIT1 in ERK1/2 activation, we studied the effect of GIT1 knockdown on signal transduction in HEK 293 cells (Fig. 7A), HeLa cells (Fig. 7B and C) and

phosphorylation was measured with phosphospecific ERK1/2 antibody (p-ERK1/2 [left panel]), and MEK1/2 phosphorylation was detected with phosphospecific MEK1/2 antibody (p-MEK1/2 [right panel]). Equal results for ERK1/2 and MEK1/2 were demonstrated by reprobing with anti-ERK1/2 or anti-MEK1 antibody (lower panels). (C and D) The relative increase in ERK1/2 and MEK1/2 phosphorylation levels compared to control results (B) was determined by performing quantitative densitometry as described in Materials and Methods (Θ , $P < 0.01$; $\ddagger$, $P < 0.05$ [mean $\pm$ standard error {SE}; $n = 3$]). (E) The same protocol was performed as described for panel B except that HEK 293 cells were cotransfected with HA-AT1R and stimulated with 200 nM AngII for the indicated times. (F and G) The relative increases in ERK1/2 and MEK1/2 phosphorylation compared to the control results (Fig. 6E) were determined by performing quantitative densitometry (*, $P < 0.01$ [mean $\pm$ SE; $n = 3$]). (H) Cells were transfected with Xpress-GIT1(wt) or pcDNA3 as described for panel B and stimulated with EGF for the indicated times. Activation of p38 was assayed by probing total cell lysates with anti-phosphospecific p38 (p-p38) antibody. Equal loading was confirmed by reprobing with anti-p38 antibody.

FIG. 8. PP2 inhibits AngII- and EGF-induced GIT1 phosphorylation and ERK1/2 activation. (A) HEK 293 cells were transfected with Xpress-GIT1(wt) and the AT1R (left panels) or with Xpress-GIT1(wt) alone (right panels) for 24 h and then serum starved for 16 h prior to the experiment. Cells were stimulated with 200 nM AngII for 2 min or with 10 ng of EGF/ml for 5 min. Lysates were immunoprecipitated (IP) with Xpress antibody and immunoblotted (IB) with 4G10. To confirm equal protein immunoprecipitation, the blot was reprobed with anti-Xpress antibody. (B) HEK 293 cells were transfected with Xpress-GIT1 for 24 h and then serum starved for 16 h. Cells were pretreated with 1 μ M PP2 for 30 min prior to EGF stimulation for 5 min. Cell lysates were immunoprecipitated with Xpress antibody and immunoblotted with 4G10. Equal protein immunoprecipitation was confirmed by reprobing with anti-Xpress antibody. (C) HEK 293 cells were transfected with pcDNA3, Src(Y527F), Src(Y416F), and Src(K295R) for 24 h. Cell lysates were immunoprecipitated with Xpress antibody and immunoblotted with 4G10. Equal levels of protein immunoprecipitation were confirmed by reprobing with anti-Xpress antibody. (D) HEK 293 cells were transfected with Xpress-GIT1 or pcDNA3 for 24 h and then serum starved for 16 h. Cells were treated with (+) or without (-) 1 μ M PP2 for 30 min prior to agonist. Cells were stimulated with (+) or without (-) 10 ng of EGF/ml for 10 min. ERK1/2 activation was measured with phosphospecific-ERK1/2 antibody (upper panel). The membranes were stripped and reprobed with ERK1/2 antibody to assure equal loading (lower panel).

VSMC (Fig. 7D). Because HEK 293 cells express low levels of endogenous GIT1, we cotransfected them with three RNAi constructs (see Materials and Methods) and Xpress-GIT1(wt). There was a concentration-dependent decrease in GIT1 expression for RNAi-1A, RNAi-2A, and RNAi-3A associated with a simultaneous decrease in ERK1/2 phosphorylation (Fig. 7A). RNAi-3A was the most effective inhibitor of human GIT1 expression and ERK1/2 phosphorylation. We next used HeLa cells, because they express readily detectable levels of endogenous GIT1. HeLa cells were transfected with 4 μ g of RNAi-3A or control RNAi (GFP-RNAi), and ERK1/2 activation was examined in response to the presence of EGF. GIT1 expression was decreased by 80% (Fig. 7B, bottom panel) without a change in ERK1/2 expression (Fig. 7B, middle panel). EGF-stimulated ERK1/2 activation was significantly inhibited at 5 and 10 min (decreases of 20 and 60%, respectively) (Fig. 7B, top panel, and 7C). To obtain further evidence for a critical role of GIT1 in ERK1/2 activation, we designed antisense GIT1 oligonucleotides and transfected VSMC to decrease GIT1 expression (Fig. 7D). AngII stimulation of ERK1/2 was significantly inhibited in cells transfected with antisense GIT1 oligonucleotides compared to the results seen with sense GIT1 oligonucleotides (decrease of 70%) (Fig. 7D). There was no significant decrease in ERK1/2 expression with any of the RNAi constructs or antisense oligonucleotides. In

three different cell types, thus, decreased GIT1(wt) expression is associated with significant inhibition of agonist-mediated ERK1/2 phosphorylation.

Phosphorylation of GIT1 and ERK1/2 is c-Src dependent.

Our laboratory previously showed that GIT1 was rapidly tyrosine phosphorylated in a Src-dependent manner in VSMC stimulated with AngII (38). To show that the same pathways were important in HEK 293 cells, we transfected HEK 293 cells with Xpress-GIT1(wt) and AT1R. Stimulation of these cells with AngII (200 nM, 2 min) increased GIT1 tyrosine phosphorylation threefold (Fig. 8A, left panels) to an extent similar to that observed with EGF (10 ng/ml, 5 min) (Fig. 8A, right panels). To prove that GIT1 phosphorylation was Src kinase dependent, we repeated the experiments in the presence of 10 μ M PP2, which completely inhibited EGF-mediated GIT1 tyrosine phosphorylation (Fig. 8B). We used three Src constructs to study the role of Src in GIT1 phosphorylation. (i) Src(Y527F) is a constitutively active Src. (ii) Src(Y416F) is a point mutation in Src at the activation loop that is predicted to eliminate regulated Src activity and result in a molecule with only basal activity. Src(Y416F) cannot be activated by agonists. (iii) Src(K295R) cannot bind ATP and has no basal or agonist activity. As expected, Src(Y527F) was associated with increased GIT1 phosphorylation and Src(Y416F) and Src(K295R) showed no stimulation of GIT1 phosphorylation (Fig.

FIG. 9. Effects of GIT1 tyrosine mutant (Y321F) on EGF- and AngII-induced signaling. (A) HEK 293 cells were cotransfected with pcDNA3, Xpress-tagged GIT1(wt), or GIT1(Y321F) for 24 h and then serum starved for 16 h. Cells were stimulated with 10 ng of EGF/ml for 10 min. ERK1/2 and MEK1 activation were measured with phosphospecific antibodies. The membranes were stripped and reprobed with ERK1/2 and MEK1 antibodies to assure equal loading (lower panels). GIT1 expression was confirmed with anti-Xpress antibody. (B) The same experimental procedure was performed as described for panel A except that cells were cotransfected with HA-tagged AT1R (ratio, 3:1) as described in Materials and Methods. Cells were stimulated with 200 nM AngII for 2 min (+) or left untreated (-). After preparation of cell lysates, ERK1/2 activation was measured with a phospho-ERK1/2 antibody. Equal levels of expression of GIT1(wt) and GIT1(Y321F) were assured by stripping and reprobing the membranes with Xpress antibody (lower panel).

8C). In addition, we tested whether EGF-stimulated ERK1/2 phosphorylation (mediated by GIT1) was sensitive to Src inhibition. As shown in Fig. 8D, transfection with GIT1(wt) increased EGF-stimulated pERK1/2 compared to that seen with pcDNA3. In the presence of PP2, there was complete inhibition of ERK1/2 phosphorylation. Because PP2 may inhibit other Src kinase family members, the relative contributions of Src in specific cell types remain to be determined. In sum, these data support the Src dependence of GIT1 phosphorylation and suggest that phosphorylation of GIT1 is critical for increased ERK1/2 phosphorylation.

To define further the role of tyrosine phosphorylation in GIT1 in ERK1/2 signaling, we mutated all tyrosine residues in GIT1 to phenylalanine. To investigate the effects of these mutations on ERK1/2 activity, we transfected HEK 293 cells with the GIT1 mutants and stimulated with EGF for 10 min. In cells transfected with GIT1(wt), there were significant increases in ERK1/2 and MEK1 activation compared to that seen with pcDNA3, as measured with phosphospecific antibodies (Fig. 9A). In contrast, in cells transfected with GIT1(Y321F) there was no significant difference from vector control results (Fig.

9A). No other GIT1 tyrosine mutation significantly altered ERK1/2 activation (data not shown). To confirm these results, we repeated the experiment with HEK 293 cells cotransfected with the GIT1 mutants and the AT1R (Fig. 9B). In response to AngII, ERK1/2 activity increased fourfold in cells transfected with GIT1(wt). In contrast, there was only a 1.7-fold increase in ERK1/2 activation in cells transfected with GIT1(Y321F). The residual ERK1/2 activation in response to AngII likely reflects the presence of GIT1(Y321F)-independent pathways activated by AngII (but not by EGF). Because Y321 is within the GIT1 SHD that mediates binding to MEK1, we next investigated whether this mutation altered the interaction with MEK1. Coimmunoprecipitation experiments showed that GIT1(Y321F) did not significantly decrease MEK1 binding levels (data not shown). These results suggest an important role for GIT1 Y321 phosphorylation, independent of GIT1-MEK1 binding, in c-Src-mediated ERK1/2 activation.

DISCUSSION

The major finding of this study was the identification of GIT1 as a c-Src substrate that mediates agonist-dependent activation of MEK1 and ERK1/2. GIT1 appears to function as a scaffold for the MEK1-ERK1/2 pathway (since it increases activation of this pathway), is specific for this pathway (no effect on p38), and binds MEK1. To our knowledge, GIT1 is the first tyrosine phosphorylation-dependent MAP kinase scaffold to be described that is positioned at the "crossroads" of c-Src signal transduction (Fig. 10). The structural domains of GIT1 required for its scaffold function are located in the central portion (aa 250 to 474) encompassing the SHD, CC1, and CC2 domains (Fig. 7 and Table 1). ERK1/2 activation is mediated by binding of MEK1, which requires interaction with the SHD and CC2 domains of GIT1. Although binding of MEK1 to GIT1 is constitutive, it appears that specific conformational changes likely occur in GIT1 (and/or MEK1) in response to tyrosine phosphorylation of Y321 in GIT1. Phosphorylation of Y321 by AngII in VSMC was demonstrated both by a decrease in the total level of GIT1 tyrosine phosphorylation in cells transfected with GIT1(Y321F) and by the disappearance of a phosphopeptide, according to the results of mass spectrometry analysis of GIT1(Y321F) cell lysates (J. Haendeler and B. C. Berk, unpublished data).

This concept is consistent with previous studies from our laboratory in which investigations of Src^{-/-} cells and c-Src inhibitors demonstrated a correlation between decreased GIT1 tyrosine phosphorylation and ERK1/2 activity (18, 38). On the basis of the results of the present study, we propose a model to explain the role of GIT1 in activation of MEK1 and ERK1/2 by AngII and EGF (Fig. 10). In serum-starved cells, GIT1 exists in a preassembled complex with MEK1. Upon agonist binding (for both GPCRs and TKRs), c-Src is activated and phosphorylates critical tyrosine residues in GIT1 (e.g., Y321). We propose that a conformational change in GIT1 then recruits additional signaling molecules (e.g., FAK, Raf, and PAK) and/or enables c-Src to phosphorylate tyrosine residues on GIT1 that lead to activation of MEK1-ERK1/2. The reasons why GIT1(del-SHD) and GIT1(del-CC2) did not act as dominant negatives are unclear. We speculate that the effect of these mutants (which was most apparent at 10 min) reflects the

FIG. 10. Model for AT1R and EGFR regulation of GIT1, MEK1, and ERK1/2. AngII binding to the AT1R or EGF binding to the EGFR activates c-Src. c-Src phosphorylates GIT1. Conformational changes in GIT1 induced by tyrosine phosphorylation (especially at Y321) lead to recruitment of other molecules and activation of MEK1.

role of GIT1 in a subpopulation of the total MEK1-ERK1/2 population present in the cell. Because GIT1 appears to be important in function of focal adhesions, it is possible that the activation of ERK1/2 at 10 min reflects ERK1/2 that is primarily located in focal adhesions (as opposed to cytoplasm or nucleus). For example, Slack-Davis et al. (41) showed that pERK1/2 was activated in focal adhesions at 10 min in response to fibronectin but not in the nucleus.

GIT family members have been shown to have important roles in receptor endocytosis (9, 10, 29) and cell motility (48, 53). Study reports from Zhao et al. (53) and from Turner and colleagues (43, 48) indicate an important role for GIT family members in focal adhesions and cell motility. Specifically, these authors propose that upon cell activation, cdc42 recruitment of PAK and PIX drives the association of GIT1 with focal adhesions. This favors dissociation of paxillin from focal adhesions which become destabilized and promotes motility by decreasing cell adherence. The association of GIT proteins with PIX, PAK, and paxillin suggests a functional role for GIT proteins in regulation of the cytoskeleton and focal adhesions (11, 30, 53). Some of the primary signaling events that occur concomitantly with cell adhesion include the phosphorylation of FAK (17), Src-mediated tyrosine phosphorylation of adhesion proteins (46), and stimulation of MAP kinase cascade (36). Recent work suggests that ERK is targeted to newly formed sites of cell-matrix interaction by integrin and c-Src activation (14). Thus, a potential function of GIT1 is to regulate ERK localization and/or activation at focal adhesions, which may be important for migration.

The information regarding GIT1 structure function generated in this study further supports a role for GIT1 in cell shape and migration. Judged on the basis of current information regarding sequences and motifs, only GIT1 and the yeast protein Spa2p, which is important for polarized morphogenesis, contain a conserved SHD (Spa2p homology domain). The SHD present in Spa2p has been shown to bind Ste11p (a MEK kinase) and Ste7p (a MEK) and appears to promote polarized morphogenesis through regulation of the actin cytoskeleton and signaling pathways (44). We speculate that the SHD of Spa2p is important for both mating and MAP kinase signaling

by operating to integrate cytoskeleton and MAP kinase pathways. The SHD domain of GIT1 binds PIX and FAK (43, 48, 53), both of which are enriched in Cdc42/Rac focal complexes, as well as MEK1, as shown in the present study. PAK-PIX-GIT1-paxillin complexes are thought to play an important role in cell lamellipodia and migration (7, 48). It is possible that MEK1-ERK1/2 localizes to focal adhesions in part via association with GIT1. This concept is in agreement with data regarding activation and localization of ERK1/2 to lamellipodia and focal adhesions during cell spreading and inhibition of cell spreading by MEK1 inhibitors and dominant-negative MEK1 (14, 20). Therefore, we propose that GIT1 might serve to link processes involved in focal adhesion formation and disassembly with MAP kinase signaling.

Key issues in MAP kinase signaling are the mechanisms that regulate spatiotemporal activation as well as signal transduction specificity and amplification. In yeast, the STE5 protein functions as a scaffold that organizes the three components of a pheromone-responsive MAP kinase cascade into a module. Homology searches and other techniques have led to discovery of other scaffolds for the ERK and JNK pathways, including KSR1 (MEK1-ERK1/2) (27, 51), MP1 and its binding partner P14 (MEK1-ERK1/2) (34, 50), MKP1 (MEK1-ERK1/2) (28), JIP1 (MKK4-JNK) (49), JSAP1/JIP3 (ASK1-MKK4/MKK7-JNK3) (25), SKRP1 (ASK1-MKK7-JNK) (52), β -arrestin (ASK1-MKK4-JNK) (23), and IB2/JIP2 (MLK3-MKK3-p38) (8). We propose that GIT1 be added to this list as a MEK1-ERK1/2 scaffold that regulates activation at specific intracellular sites such as focal adhesions and actin cytoskeleton.

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