GIT1 Is a Scaffold for ERK1/2 Activation in Focal Adhesions*

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GIT1 (G protein-coupled receptor kinase-interacting protein 1) has been shown to regulate focal adhesion disassembly. We previously reported that GIT1 associates with MEK1 and acts as a scaffold to enhance ERK1/2 activation. Here, we show that GIT1 co-localizes with ERK1/2 in focal adhesions and regulates cell migration in vascular smooth muscle cells, HEK293 cells, and HeLa cells. Immunofluorescence showed that GIT1 co-localized with phospho-ERK1/2 in focal adhesions after epidermal growth factor stimulation. Because Src is required for both GIT1 tyrosine phosphorylation and focal adhesion disassembly, we studied the effects of Src on GIT1-ERK1/2 interactions. PP2 (4-amino-5-(4-chlorophenyl)-7-[(4-butyl)pyrazolo[3,4-d]pyrimidine) inhibited association of GIT1 with ERK1/2, and their co-localization in focal adhesions was dramatically decreased in SYF−/− cells. GIT1 small interfering RNA significantly inhibited ERK1/2 recruitment to and activation in focal adhesions. GIT1 small interfering RNA and mutated GIT1 lacking the MEK1 binding domain significantly decreased epidermal growth factor-stimulated cell spreading and migration, suggesting that GIT1-mediated events such as ERK1/2 activation are required for spreading and migration. In summary, the present study further supports a key role for GIT1 (a MEK1-binding protein) as a scaffold for signal transduction in focal adhesions.

Cell motility requires coordinated temporal and spatial regulation of cell extension, adhesion, deadhesion, and contraction. The epidermal growth factor receptor is a model transmembrane tyrosine kinase-coupled receptor that mediates cell migration as one of its many cell effects. It has been suggested that such specific cellular responses are determined by the spatial targeting of downstream signaling events. This simple concept is complicated by the fact that epidermal growth factor receptor-mediated cell motility requires signaling through the ubiquitous intracellular kinases ERK1/2,‡ which are present in several compartments including cell membrane, focal adhesions, and nucleus (1, 2). The MAPK pathway (Raf/MEK1/ERK1/2) has been shown to stimulate the intracellular motility machinery independently of de novo gene transcription (1). For example, ERK1/2 has been shown to activate the ubiquitous intracellular protease calpain to promote cell death (1). Furthermore, Src-induced formation of the calpain–FASK/ERK1/2 complex and ERK1/2 activation are required for calpain-mediated proteolysis of FAK (3). Evidence shows that ERK1/2 enhances cell migration via regulation of myosin light chain kinase and myosin light chain phosphorylation (4, 5), both of which promote cytoskeletal contraction. However, little is currently known about the mechanisms that regulate spatial organization of ERK1/2 required for cell migration.

GIT1 (also termed Cat-1 for Cool (cloned out of library)-associated tyrosine phosphorylation protein) was originally identified in a yeast two-hybrid screen for proteins that bound G protein-coupled receptor kinase (GRK2) (6). Initial results demonstrated that GIT1 functions as a GTPase-activating protein for the ADP-riboseylation factor family of proteins. The two GIT family members (GIT1 and GIT2 (also termed PKL)) have been shown to have important roles in receptor endocytosis (6–8) and cell motility (9, 10). GIT1 has several domains (10) including an amino-terminal ADP-ribosylation factor–GTPase-activating protein domain, an ankyrin-rich repeat, a SHD-1 domain, and a tyrosine-rich domain. The SHD-1 domain is named for the yeast Spa1 homolog domain and spans amino acids 255–365. A linker of unknown function connects the amino terminus to several coiled-coil (CC) domains in the carboxyl terminus. There are three putative CC domains in GIT1: CC1 (residues 254–274), CC2 (residues 426–474), and CC3 (residues 659–689). GIT1 associates with many proteins including CC2 domain interactions with PAK (11); SHD-1 domain interactions with FAK, PIX, phospholipase C-γ, and MEK1; and carboxyl terminus interactions with paxillin (10, 12, 13). Studies from Zhao et al. (10) and Turner and colleagues (9, 11) indicate an important role for GIT family members in focal adhesion assembly and cell motility. Specifically, these authors propose that upon cell activation, Cdc42 recruitment of PAK and PIX drives association of GIT1 with focal adhesions. This favors dissociation of paxillin, which destabilizes focal adhesions, and promotes motility by decreasing cell adherence. The association of GIT1 with PAK, PX, and paxillin suggests a functional role in the regulation of cytoskeleton and focal adhesions (10, 14, 15).

Recently, we demonstrated that GIT1 associates with MEK1 through the SHD-1 domain (13). The SHD domain in the yeast Spa1II homolog domain promotes polarized morphogenesis in yeast through regulation of the actin cytoskeleton and polarity signaling pathway (16). Thus, a function of GIT1 may be to

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The abbreviations used are: ERK, extracellular signal-regulated kinase; pERK, phospho-ERK; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; EGFR, epidermal growth factor; FAK, focal adhesion kinase; CC, coiled-coil; VSMC, vascular smooth muscle cell; DMEU, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin; GST, glutathione S-transferase; RNAi, RNA interference; Mab, monoclonal antibody; WT, wild type; GFP, green fluorescent protein; JNK, c-Jun NH2-terminal kinase; PAK, p21-activated protein kinase; PIX, Pak-interacting exchange factor; PP2, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine.
regulate MEK1/ERK1/2 localization and activation at focal adhesions. Here, we provide evidence that GIT1 associates with ERK1/2 in focal adhesions and recruits additional ERK1/2 to focal adhesions after EGF stimulation via a Src-dependent pathway that stimulates cell migration.

MATERIALS AND METHODS

Cell Culture—Vascular smooth muscle cells (VSMCs) prepared as described previously (17) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in 5% CO₂. HEK293 cells were transfected with Lipofectamine (Invitrogen). HeLa cells were transfected with Lipofectamine 2000 (Invitrogen). SYF−/− and SYF+/+ cell lines derived from src−/− and src+/+ mice embryos, respectively, were a generous gift from Jonathan A. Cooper (University of Washington) and cultured in DMEM supplemented with 10% fetal bovine serum and 50 μg/ml G418.

DNA Expression Plasmids—Full-length mouse GIT1 cDNA and GIT1 mutation constructs were described previously (12). GIT1 wild type and GIT1 residues 590–770 (amino acids 590–770) were subcloned into a pEGFP-C2 vector using EcoRI and Sall. The PCR product of GIT1 (del CC2 lacking amino acids 420–475), was ligated with pEGFP-C2 using EcoRI and Sall, and with pCMC-DEST (Invitrogen) using EcoRI and XmaI. The reaction mixture was transformed into a suitable Escherichia coli host to select for the correct clone. Next, pAd/PL-Dest (Invitrogen) was digested with EcoO1091 (CFP-GIT1-RNAi and CFP-GFP-RNAi).

Oligonucleotides encoding short RNA hairpins targeted against conserved regions of human (NM_140330) and mouse (XM_126291) GIT1 (13) were annealed and cloned into BseRI/BamHI-cut pSHAG just downstream of the U6 promoter. We compared three different RNAi sequences based on our previous publication (13). We used construct 3, oligonucleotides 3A (5-CTC-CAT-GTA-CTC-CTG-CAG-CAT-CAG-C-CGA-AGT-TTG-GTC-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-G TG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GTG-GT
GIT1 Knockdown Inhibited ERK1/2 Translocalization and Phosphorylation in Focal Adhesions—To define the functional relationship between GIT1 and ERK1/2, we studied the effect of GIT1 knockdown on pERK1/2 and ERK1/2 localization. We previously reported that GIT1 RNAi inhibited endogenous GIT1 expression by $-85\%$ in HeLa cells (13). In HeLa cells transfected with GFP-RNAi (control), pERK1/2 localized to focal adhesions and the nucleus after EGF stimulation (Fig. 2, arrows). EGF stimulation led to an increase in total ERK1/2 in focal adhesions (Fig. 3f, arrows). In contrast, localization of pERK1/2 and ERK1/2 in focal adhesions was significantly inhibited by transfection with GIT1 RNAi (Fig. 2, d and h). Importantly, localization of pERK1/2 to the nucleus was not inhibited by GIT1 knockdown (Fig. 2, d and h). These results indicate that GIT1 is important for pERK1/2 and ERK1/2 localization in focal adhesions. GIT1-RNAi did not grossly affect the structure of focal adhesions as shown by cell morphology and by paxillin and vinculin localization (Fig. 2, i–n). These data are similar to those we previously obtained for endothelial cells, in which GIT1 knockdown did not alter basal cell morphology but increased cell rounding and focal adhesion formation in response to thrombin (13, 19).

GIT1 Is Required for ERK1/2 Phosphorylation in Cytoskeletal Fractions—We previously reported that GIT1 knockdown decreased ERK1/2 activation by EGF that was most significant at 10 min (see Fig. 6 in Ref. 13). We studied the effect of GIT1 knockdown on ERK1/2 activation in the cytoskeleton fractions of HeLa cells to characterize further the specificity of GIT1 scaffolding. We have previously shown that this cell fractionation protocol enriches vinculin and FAK by 5–10-fold compared with total cell lysates. GIT1 expression was decreased by 80% with GIT1-RNAi in total cell lysates (Fig. 3a, Cytoskeleton, panel 4) with no change in vinculin expression in cytoskeletal fractions (Fig. 3a, Cytoskeleton, panel 3). Prior to EGF stimulation, there were no differences in ERK1/2, pERK1/2, or vinculin expression in cytoskeletal or cytoplasmic fractions (Fig. 3a, panels 1 and 4). In response to EGF, pERK1/2 was significantly increased in cytoskeletal and cytoplasmic fractions at both 5 and 10 min in cells transfected with GFP-RNAi (Fig. 3a, panels 1 and 5). Quantification showed a $-5$-fold increase (Fig. 3b). In cells transfected with GIT1-RNAi, there was a dramatic decrease in pERK1/2 only in the cytoskeleton fractions (Fig. 3a,
HEK293 cells were co-transfected with the indicated plasmids. Cell lysates were immunoprecipitated with HA antibody and probed with FLAG antibody. All constructs (GIT1-(del CC2), GIT1-(1–635), and GIT1-(250–770)) bound to ERK2. Probing with HA antibody showed equal HA-ERK2 expression and equal loading. TCL, total cell lysates. B, cells were transfected as indicated, and cell lysates were immunoprecipitated with GFP antibody and probed with HA antibody to detect HA-ERK2. The blots were reprobed with GFP antibody to confirm GIT1 expression. Probing with HA antibody showed equal HA-ERK2 expression in total cell lysates.

compare lanes 5 and 6 in panel 1 to lanes 2 and 3). There was no change in pERK1/2 in the cytoplasmic fraction (Fig. 3A, panel 5). In several experiments we observed an apparent decrease in total ERK1/2 present in cytoskeleton after treatment with GIT1-RNAi (Fig. 3A, panel 2, compare lanes 5 and 6 with lanes 1–4). Although the mechanism is unclear, the most likely explanation is increased ERK1/2 in nuclear fractions because we saw no change in cytoplasmic (Fig. 3A, panel 6) or membrane fractions (data not shown). To account for the decrease in total ERK1/2 in cytoskeleton fractions, we normalized pERK1/2 to total ERK1/2. Quantitation using this measurement showed a ~50% decrease in pERK1/2 in cytoskeleton of cells treated with GIT1-RNAi (Fig. 3B).

Phosphorylation of ERK1/2 induced by EGF was decreased in membrane fractions of cells transfected with GIT1-RNAi compared with GFP-RNAi (data not shown). There was no significant change in ERK1/2 expression in cytoplasmic and membrane fractions (Fig. 3A, panel 6; data not shown). These results demonstrate that decreased GIT1 expression is associated with significant inhibition of agonist-mediated ERK1/2 phosphorylation and location in the cytoskeleton fractions. To determine whether GIT1 modulates ERK1/2 activation in the nucleus, we used the Elk-1 reporter gene assay. There was no significant change after GIT1 knockdown in response to EGF stimulation (data not shown).

**GIT1 Associates with ERK1/2**—We previously reported that the association of GIT1 and MEK1 enhanced MEK1/2 and ERK1/2 activation in response to EGF and angiotensin II (13). These findings suggest that GIT1 is a specific scaffold protein in the MEK1/2-ERK1/2 pathway. MEK1 interaction with ERK1/2 is required for ERK1/2 activation, and both were reported to be located in focal adhesions (20), where GIT1 also localizes. To confirm that GIT1 interacts with ERK1/2, we used HEK293 cells because of their high transfection efficiency and relatively low level expression of endogenous GIT1. We co-transfected FLAG-GIT1-(1–635), FLAG-GIT1-(del SHD), and FLAG-GIT1-(250–770) with HA-ERK2 into HEK293 cells. The HA-ERK2 associated with GIT1-(1–635), GIT1-(del SHD), and GIT1-(250–770) (Fig. 4A). The fact that all constructs co-precipitated with HA-ERK2 suggested that essential amino acids were present in a binding domain that encompassed residues 420–635. Note that these experiments were performed in the absence of EGF stimulation, so the binding of ERK2 to GIT1 is relatively low, as expected. To determine which domain of GIT1 is important for interaction with ERK2, we made a GFP-GIT1-(590–770) mutant. GFP-GIT1-(WT) and GFP-GIT1-(590–770) were co-transfected with HA-ERK2. GIT1-(WT) co-precipitated with HA-ERK2, but not with GFP-GIT1-(590–770) (Fig. 4B).

Based on this deletion mutation analysis, amino acids 420–590 of GIT1 are important for interaction with ERK2. To prove physiological interaction, we used VSMCs because of ample endogenous GIT1 and ERK1/2. Endogenous GIT1 interacted with endogenous ERK2 (data not shown). A similar result was observed for ERK1 (data not shown). We selected ERK2 to determine its binding domain in GIT1.

**Specific Domains of GIT1 Mediate Interaction with ERK2**—CC structures are believed to be sites of protein-protein interaction. Three putative CC domains exist in GIT1 (10). The CC1 (amino acids 253–274) overlaps with the SHD-1, which is required for interaction with MEK1 (13), but not for interaction with ERK1/2. GIT1 residues 420–590 that are required for binding ERK1/2 overlap with the CC2 (amino acids 424–474) domain. To determine whether the CC2 region is necessary for interaction with ERK2, we co-transfected HA-ERK2 with FLAG-GIT1-(WT) and FLAG-GIT1-(del CC2). Whereas FLAG-GIT1-(WT) co-immunoprecipitated with HA-ERK2, FLAG-GIT1-(del CC2) did not (Fig. 5A). To confirm the result, we used a GST pull-down assay. As shown in Fig. 5B, FLAG-GIT1-(WT) was pulled down by GST-ERK2, but FLAG-GIT1-(del CC2) was not. Taken together, these data suggest that the CC2 domain is essential for the interaction of GIT1 with ERK2.

Because we previously reported that the CC2 domain is important for GIT1 association with MEK1 (13), we tested the effects of GIT1-(del CC2) on the MEK1-ERK1/2 pathway. We transfected HEK293 cells with GIT1-(WT) or GIT1-(del CC2) and stimulated cells with 10 ng/ml EGF. In cells transfected
for 30 min and then stimulated with EGF. The association of this increase was completely inhibited by PP2 treatment (Fig. 7D).

There was a significant decrease in the interaction between GIT1 and ERK2 (Fig. 7C, middle lane). These results suggested that Src kinase activity is required for GIT1 association with ERK1/2 in focal adhesions. To further prove this hypothesis, we used SYF/−/− and SYF/+/+ cells to study pERK1/2 levels after EGF stimulation. In SYF/+/+ cells, pERK1/2 (Fig. 7D, d, arrows) was present in focal adhesions, where it co-localized with GIT1 (Fig. 7D, e and f, arrows) after EGF stimulation. In SYF/−/− cells, pERK1/2 (Fig. 7D, j) was inhibited in focal adhesions, but GIT1 still localized to focal adhesions (Fig. 7D, k), and pERK1/2 localized to the nucleus (Fig. 7D, j). As anticipated, the co-localization of GIT1 with pERK1/2 in SYF/−/− cells was decreased after EGF stimulation (Fig. 7D, l). We found significant inhibition of pERK1/2 on Western blot in SYF/−/− cells, suggesting a critical role for these kinases in EGF signaling (data not shown). Taken together, these data suggested that Src kinase activity is required for recruitment of ERK1/2 to focal adhesions, which is dependent on association with GIT1.

Functional Effects of GIT1-ERK1/2 Interaction—GIT1, a substrate for Src kinase, is involved in disassembly of Rac/Cdc42-dependent focal adhesions that are formed during cell spreading and migration (9, 11, 13). ERK1/2 activation has also been linked to cell migration and chemotaxis (23). To investigate further the role of GIT1 association with ERK1/2 in cell migration, we transfected HeLa cells with GFP-RNAi and GIT1-RNAi. A scratch assay was performed, and cell migration was detected by 4′,6-diamidino-2-phenylindole stain. Migration was dramatically inhibited in cells transfected with GIT1-RNAi (data not shown). To demonstrate further the role of GIT1 interaction with ERK1/2 in cell migration, we prepared an adenovirus expressing GIT1-RNAi and GFP-RNAi, and we measured migration of VSMCs (Fig. 8A) and HeLa cells (Fig. 8B) using a Boyden chamber. After infection with GIT1-RNAi adenovirus at a multiplicity of infection of 50, endogenous GIT1 was decreased by 80–90%, whereas GFP-RNAi infection had no significant effect on GIT1 expression or migration compared with non-transfected cells. Cells infected with GIT1-RNAi were seeded in the upper chamber of the migration apparatus, and EGF was added to the lower chamber. As shown in Fig. 8, EGF significantly increased migration relative to serum-free medium in the presence of GFP-RNAi. In contrast, cell migration was dramatically inhibited by GIT1-RNAi adenovirus infection (Fig. 8, VSMCs = 41% of control and HeLa cells = 36% of control, p < 0.01). These results suggest an important role for GIT1 in agonist-stimulated cell migration.

**Discussion**

The major findings of this study are that GIT1 interaction with ERK1/2 is required for ERK1/2 activation in focal adhesions and may be required for cell migration. Our results demonstrate that GIT1 is a pathway-specific (MEK1-ERK1/2) and location-specific (focal adhesions) scaffold that regulates MAPK signal transduction. EGF stimulation increased ERK1/2 in focal adhesions, which was dependent on association with GIT1 and required for agonist-mediated ERK1/2 activation in focal adhesions. We previously reported that GIT1 binds MEK1 and increases both MEK1 and ERK1/2 activation. Here, we provide evidence that GIT1 directly associates with ERK1/2 in focal adhesions. Below we discuss the concept that GIT1 is a location-specific scaffold for ERK1/2 based on domain-specific binding (SHD-1 for MEK1 and CC2 domain for ERK1/2), pathway-specific activation (ERK1/2, not p38 and JNK), and spatially discrete activation (focal adhesions, not plasma membrane or nucleus). Based on the present study, we propose a model to explain the role of GIT1 in EGF-mediated activation of MEK1-ERK1/2 in focal adhesions and cell migration. In serum-starved cells, GIT1 exists in a pre-assembled complex with...
MEK-ERK1/2. Upon EGF binding, Src is activated and phosphorylates critical tyrosine residues in GIT1 (12, 22). GIT1 tyrosine phosphorylation enhances the ability of GIT1 both to associate with ERK1/2 and to increase ERK1/2 phosphorylation in focal adhesions. GIT1-(del CC2), which cannot bind ERK2, appears to act as a dominant negative to block activated ERK1/2 localization in focal adhesions (Fig. 6). Additional components of the GIT1 signaling complex in focal adhesions likely include PAK, PIX, paxillin, and FAK, based on published data (10, 12, 13, 18, 24).

Our findings demonstrate that GIT1 serves as a scaffold for MAPK activation, similar to the yeast protein Ste5p and the mammalian proteins KSR, MP1, and Sef. KSR, a scaffold for the MEK-ERK1/2 pathway, transports MEK1 from the cytoplasm to the plasma membrane and organizes Raf-1, MEK1, and ERK1/2 for efficient activation in response to membrane signaling events (25, 26). MP1 is a MAPK scaffold specifically targeted to endosomes by the adaptor P14 (27, 28). A recent study (29) showed that Sef acts as a MEK-ERK1/2 scaffold localized to the Golgi apparatus. We propose that GIT1 be added to this list as a MEK1-ERK1/2 scaffold that regulates activation in focal adhesions. Like KSR, GIT1 constitutively binds MEK1, but there is increased interaction with ERK1/2 following agonist stimulation. The observations that interruption of GIT1 scaffolding function does not affect EGF-stimulated ERK1/2 activation in the cytoplasm (Fig. 3), ERK1/2 translocation to the nucleus (Figs. 2 and 6), and nuclear ERK1/2 activation (data not shown) suggest that the scaffolding function of GIT1 is specific for ERK1/2 activation at focal adhesions. Therefore, other scaffolds (such as KSR, MP1, and Sef) likely regulate ERK1/2 activation at other sites within the cell (26). Targeting by these different scaffolds addresses the fundamental question of how a ubiquitous signaling pathway such as ERK1/2 can direct divergent cellular responses depending on the stimulus and subcellular localization.

The structural domains of GIT1 required for its scaffold function are located in the central portion (amino acids 250–474) encompassing the SHD-1 for MEK1 and the CC2 domain for ERK2 interaction, respectively. Both of these domains are required for ERK1/2 phosphorylation. Recently, Fincham et al. (2) showed that activated ERK1/2 co-localized with paxillin after fibronectin treatment and localization of activated ERK to focal adhesions was Src-dependent. The results presented in this study indicate that Src is required for ERK1/2 activation and localization in focal adhesions (Fig. 7). This concept is consistent with previous studies from our laboratory showing decreased GIT1 tyrosine phosphorylation and ERK1/2 activity in Src−/− cells and with c-Src inhibitors (22, 30).

Studies from Zhao et al. (10) and from Turner and colleagues (9, 11) indicated an important role for GIT1 and GIT2 (PKL) in focal adhesions and cell motility. Specifically, these authors proposed that upon cell activation, Cdc42 recruitment of PAK and PIX drives the association of GIT1 with focal adhesions. This interaction favours dissociation of paxillin from focal adhesions that become destabilized and promotes motility by decreasing cell adhesion (10, 14, 15). Recent reports implicate PAK as an important regulator of focal adhesion-dependent

**Fig. 7.** Src is required for ERK1/2 phosphorylation in focal adhesions. A, HEK293 cells were transfected with cDNAs as indicated for 24 h and starved overnight. Cells were pretreated with Me2SO and PP2 for 30 min and stimulated with EGF as indicated. The association of HA-ERK2 and FLAG-GIT1 was assayed by immunoprecipitation with an HA antibody and immunoblotted with FLAG antibody (top panel). Equal amounts of ERK2 were precipitated as shown by probing with HA antibody (bottom panel). B, the relative increase in association between GIT1 and ERK2 compared with control (time 0, Me2SO) was determined by performing quantitative densitometry (*, p<0.01; mean ± S.E.; n = 3). C, HEK293 cells were transfected with the indicated cDNAs for 24 h. The cell lysates were immunoprecipitated with HA antibody and probed with FLAG antibody to detect FLAG-GIT1 (top panel) and reprobed with HA antibody to confirm HA-ERK2 expression (second panel). Src (third panel) and FLAG-GIT1 (bottom panel) were measured in total cell lysates (TCL). D, SYF+/− (a−l) and SYF+/+ (g−l) cells were starved overnight and stimulated with buffer or EGF for 10 min. The cells were double-stained for pERK1/2 (a, d, g, and j) and GIT1 (b, e, h, and k). c, f, i, and l are the merged images of a and b, d and e, g and h, and j and k, respectively (arrows indicate pERK1/2).
ERK1/2 activation through regulation of the upstream kinases Raf and MEK, both of which have been identified as points of convergence between cell adhesion and growth factors. Overexpression of GIT1 increases PAK activation and targets constitutively activated PAK to focal adhesions via its interaction with PIX (18, 24). Based on the present study, it appears likely that pathway-specific scaffold proteins control the relative activation of JNK (e.g. JIP-1) and ERK1/2 (e.g. GIT1) in focal adhesions. We speculate that the relative abundance of these scaffolds and the kinases that regulate their conformation and binding properties determines the magnitude of specific MAPK activation in specific locations.

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FIG. 8. GIT1 is required for cell migration. VSMCs (A) or HeLa cells (B) were infected with GIT1-RNAi or GFP-RNAi adenovirus for 8 h and then changed to normal medium and incubated for 36 h. Cells were starved overnight and then seeded in the upper Boyden chamber on collagen-precoated PVP-free polycarbonate membranes. EGF (20 ng/ml) was added to the lower chamber. Cells were incubated for 6 h at 37 °C in a 95% air/5% CO2 humidified incubator. The membranes were then washed, and cytoskeletal components, as well as transcription factors. Among cytoskeletal proteins, myosin light chain kinase, calpain, FAK, and paxillin are most likely to be involved in ERK1/2-mediated cell migration. Activated ERK1/2 promotes phosphorylation of myosin light chain kinase and myosin light chain, which may be involved in focal adhesion turnover and membrane protrusion at the front of polarized cells (5). ERK1/2 phosphorylates m-calpain at Ser-50, which is required for focal adhesion turnover and cell migration (32). ERK1/2 also phosphorylates FAK at Ser-910, which inhibits its interaction with paxillin (33). Liu et al. (34) have shown that ERK1/2 phosphorylates paxillin in hepatocyte growth factor-stimulated epithelial cells and that paxillin phosphorylation in turn enhances paxillin-FAK association. These intriguing observations suggest a sophisticated regulation of the FAK-paxillin complex by ERK1/2.
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