Identification of phosphorylation sites in GIT1

Donna J. Webb[1,*,‡,§], Mark W. Mayhew[1,*], Mykola Kovalenko[1,*], Melanie J. Schroeder[2,*], Erin D. Jeffery[2,*], Leanna Whitmore[1], Jeffrey Shabanowitz[2], Donald F. Hunt[2,3] and Alan F. Horwitz[1]

Departments of [1]Cell Biology, [2]Chemistry, and [3]Pathology, University of Virginia, Charlottesville, VA 22908, USA

*These authors contributed equally to this work
‡Present address: Department of Biological Sciences and Vanderbilt Kennedy Center for Research on Human Development, Vanderbilt University, Nashville, TN 37235, USA
§Author for correspondence (e-mail: donna.webb@vanderbilt.edu)

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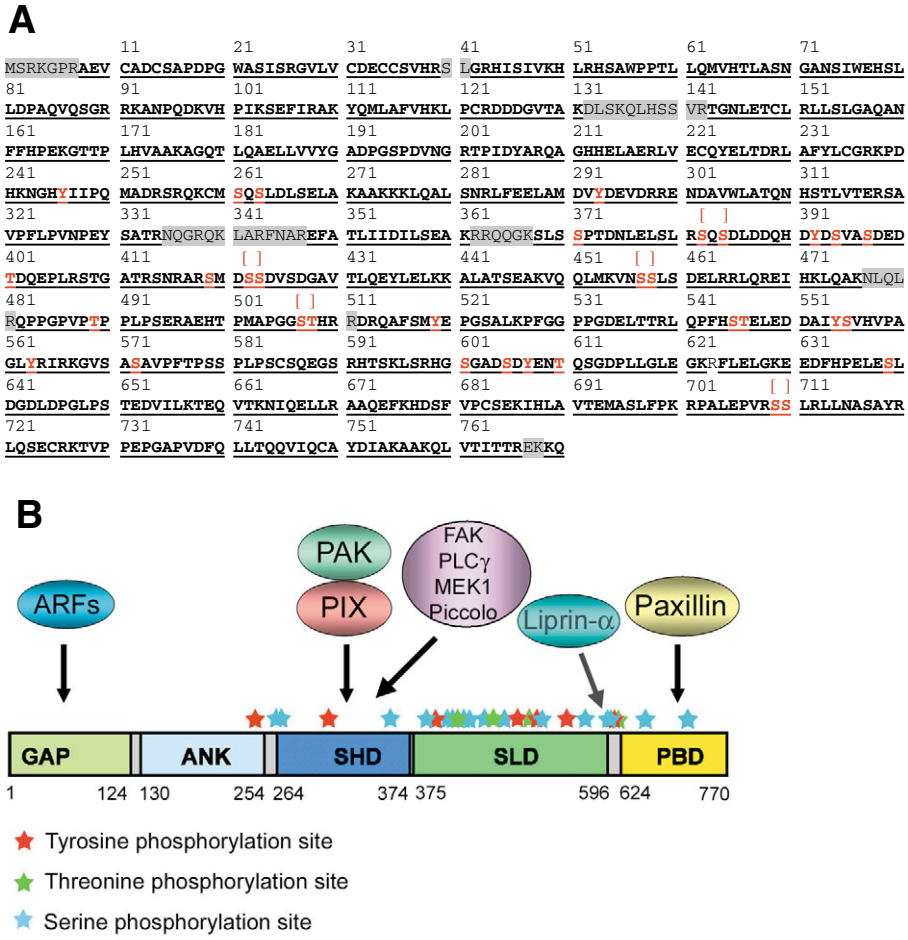

Fig. 1. Phosphorylation sites detected in human GIT1. (A) Ser, Thr and Tyr coverage of the FLAG-GIT1 sequence (tag not shown) generated with trypsin. Detected tryptic peptides are bold and underlined. Residues not covered are shaded in gray. Observed phosphorylation sites are red. Red brackets above residues indicate that a phosphorylation site could not be located unambiguously to a specific amino acid. Ninety-six percent of the Ser, Thr and Tyr sites were covered. (B) Illustration of GIT1 with its domains. GIT1 contains a N-terminal ARF-GAP domain, ankyrin repeats (ANK), a Spa2 homology domain 1 (SHD1) and a C-terminal paxillin-binding domain. GIT1 localizes to synapses in hippocampal neurons via a newly described domain termed synaptic localization domain (SLD). Several signaling molecules, including focal adhesion kinase (FAK), p21-activated kinase interacting exchange factor (PIX), phospholipase Cγ (PLCγ), mitogen-activated protein kinase kinase 1 (MEK1) and the synaptic protein Piccolo, associate with GIT1 through SHD (Haendeler et al., 2003; Kim et al., 2003; Yin et al., 2004; Zhao et al., 2000). The synaptic protein liprin-α interacts with GIT1 through a region that includes part of the SLD and the C-terminus of GIT (Ko et al., 2003). Stars indicate the approximate locations of phosphorylation sites within the various protein domains.

G protein-coupled receptor kinase-interacting protein 1 (GIT1) was originally identified as an ADP ribosylation factor GTPase-activating protein (ARF-GAP) that binds G-protein-coupled receptor kinases (GRKs) and regulates membrane trafficking (Premont et al., 1998). Subsequent studies have shown a much broader function for GIT1 and GIT2/PKL as regulators of migration-related processes, including adhesion and cytoskeletal organization (Manabe et al., 2002; Mazaki et al., 2001; West et al., 2001; Zhao et al., 2000). GIT function and localization are most likely mediated through its interaction with various signaling molecules, including paxillin, p21-activated kinase interacting exchange factor (PIX), focal adhesion kinase (FAK), phospholipase Cγ (PLCγ) and mitogen-activated protein kinase kinase 1 (MEK1) (Bagrodia et al., 1999; Haendeler et al., 2003; Manabe et al., 2002; West et al., 2001; Yin et al., 2004; Zhao et al., 2000). In fibroblasts and epithelial cells, GIT1 regulates migration and protrusive activity by assembling and targeting multi-protein signaling complexes that contain actin regulators, such as PIX and the Rac/Cdc42 effector p21-activated kinase (PAK), to adhesions and the leading edge of a protrusion (Di Cesare et al., 2000; Manabe et al., 2002). Another GIT family member, PKL, which is the chicken homolog of GIT2, also recruits PIX and PAK to adhesions through its interaction with paxillin (Brown et al., 2002). Once in adhesions, GIT1 promotes their disassembly through a PIX-dependent mechanism and stimulates motility (Zhao et al., 2000).

The interaction of GIT1 with signaling molecules is mediated through a series of domains, including an N-terminal ARF-GAP domain, ankyrin repeats (ANK), a PIX-binding domain (Spa2 homology domain 1, SHD1) and a C-terminal paxillin-binding domain (PBS) (Manabe et al., 2002; West et al., 2001). Although initial studies showed that the ARF-GAP domain of GIT1 regulates receptor endocytosis, it has been subsequently shown to function in other capacities (Premont et al., 1998). The ARF-GAP domain is crucial for the GIT1-stimulated activation of PAK but, interestingly, the GAP activity is not (Loo et al., 2004). The regulation of polarized Rac activity and cell spreading by an α4-integrin–paxillin–GIT1 complex requires the ARF-GAP domain (Nishiya et al., 2005). The SHD domain serves as a binding site for several signaling molecules,

Table 1. Summary of GIT1 phosphorylation sites

Sites	Detected without enrichment* (trypsin)	Detected with calyculinA†	Homologs/other family members	Putative kinases‡
Y246	IMAC		mGIT1 and rGIT1	EGFR
S261	+	+	mGIT1 and rGIT1	
S263	+	+	mGIT1, rGIT1 and GIT2	
Y293	+		mGIT1, rGIT1 and GIT2	Src, Lck, EGFR
S371	+		mGIT1 and rGIT1	p38MAPK
S382/S384	+		mGIT1, rGIT1 and GIT2	
Y392	+		mGIT1, rGIT1 and GIT2	
S394	+		mGIT1, rGIT1 and GIT2	
S397	+		mGIT1, rGIT1 and GIT2	
T401	+		mGIT1, rGIT1 and GIT2	
S419	+		mGIT1, rGIT1 and GIT2	CaMII, Akt
S422/S423	IMAC		mGIT1 and rGIT1	
S457/S458	IMAC		mGIT1 and rGIT1	PKA (S458)
T489	+		GIT2	p38MAPK, GSK3
S507/T508	IMAC		mGIT1 and rGIT1	PKC (T508)
Y519	+		mGIT1, rGIT1 and GIT2	
S545	IMAC		mGIT1 and rGIT1	
T546	IMAC	+	mGIT1 and rGIT1	
Y554	+		mGIT1 and rGIT1	Src
S555	+	+	mGIT1 and rGIT1	
Y563	IMAC		mGIT1 and rGIT1	
S572	IMAC		mGIT1, rGIT1 and GIT2	
S601	+		mGIT1, rGIT1 and GIT2	PKC, PKA, GSK3
S605	+		mGIT1, rGIT1 and GIT2	
Y607	+		mGIT1, rGIT1 and GIT2	EGFR
T610	+	+	mGIT1, rGIT1 and GIT2	
S639	+		mGIT1 and rGIT1	
S709/S710	IMAC		mGIT1 and rGIT1	PKA (S710)

Shown are phosphorylation sites of human GIT1 detected by mass spectrometry [the numbering is according to our GIT1 sequence, which has a nine-amino-acid insertion following amino acid 253 from the rat GIT1 sequence as described in Manabe et al. (Manabe et al., 2002)]. The exon nucleotide sequence (AGA TCT CGG CAA AAG TGC ATG TCT CAG) for this insertion is embedded at the 3′ end of intron 7 to intron 8 of the GIT1 gene, prior to exon 8, and could represent a splice variant; m, mouse; r, rat.

*Phosphorylation sites detected without enrichment are denoted with a '+'. Sites labeled by IMAC were only detected after sample enrichment by immobilized metal affinity chromatography.

†These sites were detected upon addition of calyculin A to peroxovanadate-treated cells.

‡Kinases predicted by NetPhos 2.0 and Scansite to phosphorylate a particular site. Notice that this is a partial list and includes only kinases that have been implicated in cell migration.

For additional data regarding GIT1 phosphorylation, see the Cell Migration Consortium web site: http://www.cellmigration.org.

including PIX, FAK, PLCγ and MEK1 (Haendeler et al., 2003; Yin et al., 2004; Zhao et al., 2000), and for the synaptic protein Piccolo (Kim et al., 2003). This domain of GIT1 is necessary for the formation of the multi-protein cytoplasmic complexes, which function in the targeting of the signaling molecules (Manabe et al., 2002). The C-terminal region of GIT1, which contains a paxillin-binding domain, targets it to adhesions (Manabe et al., 2002; West et al., 2001).

In neurons, GIT1 localizes to synapses through a newly described domain termed synaptic localization domain (SLD) (Zhang et al., 2003). GIT1 recruits actin regulators, such as PIX and PAK, to these sites and regulates spine morphogenesis and synapse formation (Zhang et al., 2003; Zhang et al., 2005). The interaction of GIT1 with liprin-α, which is necessary for the postsynaptic targeting of AMPA receptors, has been mapped to a region that includes part of the SLD and the C-terminus of GIT (Ko et al., 2003). A coiled-coil region (amino acids 428-485) that is located within the SLD of GIT1 allows for homodimerization of GIT with itself or heterodimerization with PIX (Premont et al., 2004). This region functions in the assembly of the large, oligomeric GIT-PIX complexes (Premont et al., 2004). The coiled-coil region and the C-terminus of GIT1 mediate the interaction of GIT with huntingtin and enhance the aggregation of this protein (Goehler et al., 2004). Interestingly, large amounts of C-terminal GIT1 fragments are found in the brains of patients with Huntington's disease, which could be a significant factor in the pathogenesis of the disease (Goehler et al., 2004)

Emerging data suggests that GIT1 is regulated by phosphorylation. GIT1 becomes phosphorylated in fibroblasts upon adhesion of cells to fibronectin (Bagrodia et al., 1999). In addition, tyrosine phosphorylation of GIT1 by Src is induced by several growth factors, including EGF and PDGF (Haendeler et al., 2003). Despite the potential importance of phosphorylation in regulating GIT1 function and localization, specific phosphorylation sites within GIT1 have not been identified.

In this study, we used mass spectrometry (MS) to generate a phosphorylation map of GIT1. Immunoprecipitates of GIT1 were digested with several proteases to maximize protein coverage. The enrichment of phosphopeptides by using Fe^{3+} IMAC LC-MS/MS significantly improved our ability to detect less abundant phosphorylation sites (Ficarro et al., 2002). To minimize dephosphorylation of tyrosines, cells were incubated for 30 minutes with peroxovanadate, which inhibits tyrosine phosphatases, before they were lysed. Pretreatment with peroxovanadate dramatically increased the abundance of tyrosine-phosphorylated peptides detected by C18 LC-MS/MS. To minimize dephosphorylation of serines and threonine residues, cells were treated with both peroxovanadate and calyculin A to inhibit serine/threonine phosphatases. Under these conditions, five additional serine/threonine phosphorylation sites were detected (Table 1). The combination of these techniques enabled us to achieve 96% coverage of the protein and detect 28 phosphorylation sites (Fig. 1).

The majority of detected phosphorylation sites were concentrated in the region of GIT1 encompassing amino acids 300-600 (Fig. 1). Interestingly, at the N-terminus of the protein, no sites were detected in the ARF-GAP domain (amino acids 1-124), one site, Y246, was found at the end of the ankyrin repeats (amino acids 130-

254) and two sites, Y293 and S371, were detected in the PIX-binding domain (amino acids 264-374) (see Fig. 1A, GIT1 sequence). The SLD (amino acids 375-596) contained the majority of detected phosphorylation sites, including Y392, Y519, Y554, S555, Y563, S382/S384, S394, S397, S419, S422/423, S457/458, S507/T508, S545, S555, S572, T401, T489 and T546. The high number of detected phosphorylation sites in the SLD suggests that the function of GIT1 in synapse formation is regulated by phosphorylation. In addition, several phosphorylation sites, S639, S709 and S710, were detected in the C-terminal region of GIT1. The C-terminal part of GIT1 (amino acids 624-770), which contains a paxillin-binding domain, mediates its localization to the leading edge and adhesions, and is essential for the effects of GIT1 on cell migration and protrusion formation (Manabe et al., 2002). Many of these serines, threonines, and tyrosines are conserved in other GIT1 species (mouse and rat) and are found in another GIT family member, GIT2 (Table 1). Three GIT1 peptides were phosphorylated on multiple residues (Table 2). In two of these peptides, the corresponding residues are completely conserved among other GIT1 species (mouse and rat) and GIT2 (Table 2), suggesting that they serve a regulatory function.

Although the kinases that phosphorylate these sites have not been determined experimentally, some of these phosphorylation sites fall within predicted kinase motifs (Table 1). S458, S601 and S710 are predicted phosphorylation sites for protein kinase A (PKA), whereas S601 and T508 are potential substrates for protein kinase C (PKC) (Table 1). GSK3 is a predicted kinase for S601 and T489. Residues S371 and T489 are potential phosphorylation sites for p38 mitogen-activated protein kinase (p38MAPK), and S419 is a predicted site for Akt. Interestingly, T293 and T554 are predicted sites for Src; GIT has been shown to be phosphorylated in cells in a Src-dependent manner (Bagrodia et al., 1999). The task now is to determine whether these kinases are physiologic regulators of GIT1 phosphorylation and to establish the physiological consequences of the phosphorylations.

Table 2. GIT1 peptides that contain more than one phosphorylated residue

Sites	Position	GIT species/ homolog	Peptides
[S382/S384]/S394/S397	382-407	Human	[SQS]DLDDQHDYDSVASDEDTDQEPLR
S384/S394/S397/T401	382-407	Human	SQS DLDDQHDYDSVASDEDTDQEPLR
S384/S397	382-407	Human	SQS DLDDQHDYDSVASDEDTDQEPLR
Y392/S397	382-407	Human	SQS DLDDQHDYDSVASDEDTDQEPLR
		Mouse	SQS ELDDQHDYDSVASDEDTDQEPLP
		Rat	NQS DLDDQHDYDSVASDEDTDQEPLP
		GIT2	VES QDNDQPDYDSVASDEDTDLE---
S545/Y554	540-564	Human	LQPFHSTELEDDAIYSVHVPAGLYR
Y554/Y563	540-564	Human	LQPFHSTELEDDAIYSVHVPAGLYR
		Mouse	LQPFHSTELEDDAIYSVHVPAGLYR
		Rat	LQPFHSTELEDDAIYSVHVPAGLYR
		GIT2	LQPF---------------PAHIGR
S601/S605	599-623	Human	HGSGADSDYENTQSGDPLLGLEGKR
S601/S605/Y607	599-622	Human	HGSGADSDYENTQSGDPLLGLEGK
S605/Y607	599-622	Human	HGSGADSDYENTQSGDPLLGLEGK
		Mouse	HGSGADSDYENTQSGDPLLGLEGK
		Rat	HGSGAESDYENTQSGEPLLGLEGK
		GIT2	QNSTPESDYDNTPNDMEPDGMGS

Examples of multiply-phosphorylated GIT1 peptides (e.g. simultaneous phosphorylations on the same peptide) and their alignment with homologs and another GIT family member (GIT2). Residues in square brackets indicate that phosphorylation was detected within these residues but could not be located unambiguously to a specific amino acid.

Materials and Methods

Sample preparation

HEK cells were transfected with FLAG-GIT1 (4 μg per 100 mm dish) using lipofectamine. After 48 hours, cells were incubated with 1 mM peroxovanadate for 30 minutes and extracted with 25 mM Tris, 100 mM NaCl, 0.5% NP-40, pH 7.4. In some experiments, cells were incubated with 1 mM peroxovanadate and 5 nM calyculin A for 30 minutes, and then extracted. The lysates were precleared twice with mouse IgG-agarose for 1 hour at 4°C and immunoprecipiatated with FLAG-agarose (Sigma) for 2 hours at 4°C. Samples were washed twice with 25 mM Tris, 100 mM NaCl, pH 7.4 and FLAG-tagged GIT1 was eluted by incubating the beads with 0.2 mg/ml FLAG peptide in 25 mM Tris for 30 minutes at 4°C.

Sample analysis

Eluted samples were reduced and alkylated with dithiothreitol and iodoacetamide, respectively, as described by Schroeder et al. (Schroeder et al., 2004). Immunopurified amounts of GIT1 were estimated by visualization of the corresponding silver-stain band separated by SDS-PAGE. An aliquot of the immunopurified sample corresponding to an easily visible silver-stained band was digested with the desired enzyme (approximate ratio enzyme to substrate was 1:20) in 100 mM ammonium bicarbonate, pH 8.5 for 8-12 hours at room temperature. Generally, peptides from an aliquot that corresponded to 1-5% of the original immunoprecipitated sample were separated by reverse-phase chromatography using a 1-hour or 2-hour gradient as described by Schroeder et al. (Schroeder et al., 2004). Analysis of FLAG-eluted samples was with an LCQ XP or LTQ-FT (ThermoElectron, San Jose, CA) under conventional MS/MS mode according to Schroeder et al. (Schroeder et al., 2004). Reduction and alkylation steps were omitted for on-beads digestion (treatment with 100-500 ng of trypsin for 6 hours at room temperature (Schroeder et al., 2004) and analysis of the resulting peptides on an LTQ-FTMS). Enrichment of phosphopeptides was performed by immobilized metal affinity chromatography (IMAC) according to Ficarro et al. (Ficarro et al., 2002) by using 10-20× more sample. Phosphopeptides were eluted with 250 mM ascorbic acid.

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