G-protein-coupled Receptor Kinase-interacting Proteins Inhibit Apoptosis by Inositol 1,4,5-Triphosphate Receptor-mediated Ca\(^{2+}\) Signal Regulation*

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The inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)-R) is an intracellular IP\(_3\)-gated calcium (Ca\(^{2+}\)) release channel and plays important roles in regulation of numerous Ca\(^{2+}\)-dependent cellular responses. Many intracellular modulators and IP\(_3\)-R-binding proteins regulate the IP\(_3\)-R channel function. Here we identified G-protein-coupled receptor kinase-interacting proteins (GIT) (GIT1 and GIT2), as novel IP\(_3\)-R-binding proteins. We found that both GIT1 and GIT2 directly bind to all three subtypes of IP\(_3\)-R. The interaction was favored by the cytosolic Ca\(^{2+}\) concentration and it functionally inhibited IP\(_3\)-R activity. Knockdown of GIT induced and accelerated caspase-dependent apoptosis in both unstimulated and staurosporine-treated cells, which was attenuated by wild-type GIT1 overexpression or pharmacological inhibitors of IP\(_3\)-R, but not by a mutant form of GIT1 that abrogates the interaction. Thus, we conclude that GIT inhibits apoptosis by modulating the IP\(_3\)-R-mediated Ca\(^{2+}\) signal through a direct interaction with IP\(_3\)-R in a cytosolic Ca\(^{2+}\)-dependent manner.

The inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)-R) consisting of three subtypes, IP\(_3\)-R1, IP\(_3\)-R2, and IP\(_3\)-R3, is a tetrameric intracellular IP\(_3\)-gated calcium (Ca\(^{2+}\)) release channel localized at the endoplasmic reticulum (ER) with its NH\(_2\)-terminus and COOH-terminal tail (CTT) exposed to the cytoplasm (1, 2; see Fig. 1A). IP\(_3\)-Rs are composed of five functional domains. The long NH\(_2\)-terminal cytoplasmic region contains three domains, a coupling.suppressor domain, an IP\(_3\)-binding core domain, and an internal coupling domain. The COOH-terminal region has a six-membrane spanning channel domain and a short cytoplasmic CTT “gatekeeper domain” that is critical for IP\(_3\)-R channel opening (2, 3). Ca\(^{2+}\) release activity of the IP\(_3\)-R channel is regulated by many intracellular modulators (ATP, calmodulin, and Ca\(^{2+}\)), protein kinases, and IP\(_3\)-R-binding proteins (2, 4), and the tight regulation of IP\(_3\)-R channel activity by these factors generates various spatial and temporal intracellular Ca\(^{2+}\) patterns such as Ca\(^{2+}\) spikes and Ca\(^{2+}\) oscillations, leading to numerous cellular responses (1, 2, 5, 6).

One of the physiological roles of IP\(_3\)-mediated Ca\(^{2+}\) signaling is a pro-apoptotic regulator during apoptosis. Ca\(^{2+}\) released from ER can stimulate several key enzymes activated during apoptosis such as endonucleases (7) and calpain (8). In addition, the close proximity of ER to mitochondria may facilitate the mitochondrial overload of Ca\(^{2+}\) released from the IP\(_3\)-Rs with certain apoptotic stimuli, triggering the opening of the mitochondrial permeability transition pore and the release of apoptotic signaling molecules, such as cytochrome c and apoptosis-inducing factor, which leads to the activation of caspasases (5, 6). Moreover, several key components of apoptotic cascades, such as cytochrome c (9) and anti-apoptosis proteins Bcl-2 (10, 11) and Bcl-x\(_L\) (12), have been reported to interact with the internal coupling domain and/or the CTT of IP\(_3\)-R and enhance the Ca\(^{2+}\)-release activity of IP\(_3\)-Rs during apoptosis. In this study, we identified the ubiquitously expressed G-protein-coupled receptor kinase-interacting proteins (GIT) (13) GIT1 and GIT2, as novel IP\(_3\)-R-binding proteins that bind to the CTT of IP\(_3\)-R and inhibit apoptosis by regulation of IP\(_3\)-R-mediated Ca\(^{2+}\) signal.

EXPERIMENTAL PROCEDURES

Plasmids—All PCR products of cDNA fragments were generated in-frame using Platinum® Pfx DNA polymerase (Invitrogen). GFP-IP\(_3\)-R1 (14), GFP-IP\(_3\)-R3 (15), and monomeric red fluorescent protein (mRFP) (pcDNA4-mRFP) (16) were described previously. GFP-IP\(_3\)-R2 was generated by subcloning IP\(_3\)-R2 cDNA into the Nhel-Xbal site of pmcDNA3.1/Zeo (Invitro). The fragments encoding full-length or different partial lengths of IP\(_3\)-R1/CTT, IP\(_3\)-R2/CTT, and IP\(_3\)-R3/CTT were generated by Platinum® Pfx DNA polymerase (Invitrogen). The fragments encoding full-length or different partial lengths of IP\(_3\)-R1/CTT, IP\(_3\)-R2/CTT, and IP\(_3\)-R3/CTT were generated by Platinum® Pfx DNA polymerase (Invitrogen).
IP3R1/CTT, GST-IP3R1/GITCORE, GST-IP3R2/GITCORE, and GST-IP3R3/GITCORE. For construction of mRFP-GIT1 and mRFP-GIT2 expression vectors, the mouse GIT1 and GIT2 cDNA were amplified by reverse transcriptase-PCR using the primers: GIT1, 5'\text{/H11032}-ATTTGATATCATGTCCCGGAGGGGG-3' and 5'\text{/H11032}-ATCCTCGAGTCACTGTTTCTTCTCT-3' (based on a mouse GIT1 sequence predicted by EST; the underlines indicate the EcoRV and XhoI cloning sites, respectively, same as the followed); GIT2, 5'\text{/H11032}-ATTTGATATCATGTCGAGCGGCTCCGGAGCAGCGACGT-3' and 5'\text{/H11032}-ATCCTCGAGTCAGCTGCTGTTCTCTTTGGTGGTATT-3' (based on the reported mouse GIT2 sequence (accession number: BC056993)) and a mouse cerebellum cDNA library as the template, and they were subcloned into the EcoRV-XhoI site of pcDNA4-mRFP. The obtained mouse GIT1 cDNA represents a 761-amino acid peptide that corresponds to a reported mouse GIT1 sequence (accession number Q5F258), and the mouse GIT2 cDNA represents a 709-amino acid peptide. The cDNA encoding GIT1 was subcloned into the EcoRI-BamHI site of pFastBac1-His for the baculovirus expression system. Truncated constructs corresponding to different lengths of GIT1 were subcloned into the EcoRV-XhoI site of pcDNA4-mRFP to generate plasmids for mRFP-fused GIT1 deletion mutants (Fig. 3A), or into the EcoRI-HindIII site of pET-23a(+) (Novagen) for GIT1(416–761)-His. Site-directed mutants of GIT1 were generated using the QuikChange II site-directed mutagenesis kit (Stratagene).

Small Interfering RNAs—siRNA duplexes were purchased from B-Bridge as duplexes or a mixture of three duplexes and were originally designed for human GIT1 (accession number NM_014030) and GIT2 (accession numbers NM_139201, NM_057169, NM_014776, and NM_057170). The target sequences were: GIT1-1, 5'\text{/H11032}-CGAGGUGGAUCGAAGAGAA-3'; GIT1-2, 5'\text{/H11032}-GCACUGAGCUAGAGGACGA-3'; GIT1-3, 5'\text{/H11032}-CCAAGAACAUUCAGGAACU-3'; and GIT2, 5'\text{/H11032}-GGAAUACAGUAUGAGCUA-3'. No difference in the efficiency of knockdown of GIT1 between each of the three siRNAs and the mixture of the three siRNAs were found (data not shown). For knockdown of endogenous GIT1 in HeLa cells, a mixture of the three GIT1 siRNAs or GIT1-2 siRNA was used. For the rescue experiments, HeLa cells transfected with both GIT1-2 siRNA and mouse GIT1 cDNA, which has three substitutions for the GIT1-2 siRNA-targeted sequence (mouse GIT1 sequence, with substitutions underlined, 5'\text{/H11032}-GCACGGAGCUCGAAGACGA-3'), were used. No knockdown effect of GIT1-2 siRNA on the expressed mouse GIT1 was detected (data not shown). For

FIGURE 1. GIT1 and GIT2 bind to all three subtypes of IP3R. A, schematic representation of ER residential IP3R. The CTT of IP3R1 is used as bait in a yeast two-hybrid screen. B, schematic representation of GIT1, GIT2, and two GIT1 fragments identified from the yeast two-hybrid screen. Functional domains are indicated. ARF-GAP, ARF-specific GTPase-activating protein domain; ANK-REP, ankyrin repeats; CC, coiled-coil domains; SHD, the Spa2-homology domain; EF, EF-hand; IQ, IQ-like motifs; aa, amino acid. C, GIT1 binds to IP3R1 in vitro. GST and GST-IP3R1/CTT were incubated with mouse brain lysate for a pull-down assay. The input and pulled-down samples were probed with α-GIT1. D and E, GIT1 binds to IP3R1 in vivo. Mouse brain lysates were processed to control IgG and α-IP3R1 (D) or α-GIT1 (E) for IP. The input and IP samples were probed with α-GIT1 and α-IP3R1. F and G, both GIT1 and GIT2 bind to all three IP3R subtypes. HeLa cells coexpressing GFP-fused IP3R1, IP3R2, or IP3R3 and mRFP-fused GIT1 (F) or GIT2 (G) were processed for IP using α-RFP. The input and IP samples were blotted with α-GFP (top) and α-RFP (bottom).
GIT Proteins Inhibit Apoptosis

A. Graph showing normalized β-galactosidase activity.

B. Alignment of sequences with highlighted essential regions.

C. Western Blot (WB) of GIT1(417-761)-His protein.

D. Gel showing GST-fusion peptides and GST.

E. Graph showing probability of coiled-coil domains.

F. Schematic of GIT1 constructs.

G. Western Blot (WB) of RFP-GIT constructs.

H. Gel showing RFP-GIT constructs in pellet and input.
**GIT Proteins Inhibit Apoptosis**

**FIGURE 2.** The coiled-coil domain in the CTT of IP3R (A–E) and the IQ-like motif of GIT (F–I) are involved in the interaction. A, liquid yeast two-hybrid quantification assay of the interactions between various lengths of IP3R/GITCORE and the short GIT1 fragment identified from the yeast two-hybrid screen. Values of interaction strength were normalized against the interaction between IP3R1/CTT and GIT1 fragment. B, sequence alignment of IP3R/GITCORE (top) and schematic of IP3R/GITCORE (bottom). The boxes indicate essential residues for the binding. C and D, IP3R/GITCORE binds to GIT in vitro. The purified GIT1 (417–716)-His fragment was incubated with purified GST-fused IP3R1/GITCORE, IP3R2/GITCORE, IP3R3/GITCORE, or GST for pull-down assay. The applied and pulled-down samples were probed with α-RFP. Arrows indicate the peptides that failed to bind. E, IP3R/GITCORE overlaps the coiled-coil domain in IP3R/CTTs. The probability of coiled-coil domains for the whole length of IP3R/CTTs was predicted using the method of Lupas et al. (38).

**FIGURE 3.** Ca2+ modulates the GIT-IP3R association. A, Ca2+ enhanced the GIT-IP3R interaction in vitro. Lysates of HeLa cells expressing mRFP-GIT1 were incubated with GST-IP3R2/GITCORE in the presence of 5 mM EGTA or the indicated Ca2+ concentrations for a pull-down assay. The precipitated samples were probed with α-RFP (top) and α-GST (bottom). B and C, Ca2+ enhanced the GIT-IP3R association in vivo. After treatment with or without 10 μM ATP (B) or 1 μM TG (C) at room temperature for 10 min, HeLa cells were cultured with a membrane-permeable cross-linker, dithiobis(succinimidyl propionate), at 4°C, and then IP was performed. The input and IP samples were probed for the indicated proteins.

**Cell Culture and Transfection**—HeLa and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transfection of plasmids was performed as described previously (20). The indicated proteins were transfected using Lipofectamine 2000 (Invitrogen). Immunoprecipitation were performed as described previously (20).

**Recombinant Proteins**—Recombinant baculovirus was generated using the pFastBac1-His-GIT1 or pFastBac1-His-L712A with the Bac-to-Bac baculovirus expression system (Invitrogen). His-GIT1 and His-L712A were expressed in Sf9 cells by infecting recombinant baculovirus. GIT1-(417–761)-His and GIT-IP3R interaction were produced by immunization of Japanese white rabbits and was purified as described previously (19). Anti-IP3R2 (KM1083), and anti-IP3R3 (KM1082) were described previously (18). Anti-RFP (DSRed) and anti-GFP (Clontech), anti-caspase-3, and anti-caspase-9 (Cell signaling), anti-β-actin (Sigma), and mouse anti-GIT1 and anti-GIT2 short (BD Transduction Laboratories) antibodies were purchased. The anti-GIT2 short antibody was confirmed to recognize both GIT1 and GIT2 and used as an anti-pan-GIT antibody (see Fig. 5A). The following materials were purchased: ApoTag® Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon International), staurosporine (Sigma), Z-VAD-fmk (Promega), 2-aminoethoxydiphenyl borate (Sigma), and xestospongic C (Calbiochem).

**Yeast Two-hybrid Assay**—A yeast two-hybrid assay was performed as described previously (20). The liquid yeast two-hybrid quantification assay was performed using 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) as substrate according to the methods described in the Clontech manual.

**Pull-down and Immunoprecipitation Assay**—Pull-down and immunoprecipitation were performed as described previously (16). For Ca2+-dependent in vitro and in vivo binding assay, appropriate concentrations of EGTA and Ca2+ were added to lysis buffer and wash buffer.

**Control**—Mice cerebellar microsome fractions were incubated with purified His-GIT1 or His-L712A for 10 min on ice, and then IP3-induced Ca2+ release from microsomes was measured with Fura-2 and a fluorospectrometer, CAF110 (Jasco, Tokyo, Japan) as described previously (19). Data are presented as mean ± S.D. of at least three independent experiments.

**Ca2+ Imaging**—Ca2+ imaging was performed as described previously (19) by placing the cells in HEPES-buffered saline with or without 2 mM CaCl2. Acquisition was performed with the custom software TI Workbench. Off-line analysis was performed with TI Workbench combined with Igor Pro software (WaveMetrics). The Ca2+ response was quantitated as the area under the response curve (AUC). Data are presented as mean ± S.D. of at least five independent experiments.

**TUNEL Staining and Microscopy**—TUNEL staining was performed according to the protocol of Chemicon International. Coverslips were mounted using Vectashield mounting medium with 4’,6-diamidino-2-phenylindole (Vector Laboratories). Fluorescent images were obtained using a digital microscope.
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A

| IP3 | Control | His-GIT1 | His-L712A |
|-----|---------|----------|-----------|
| 2 nM | ![Graph](image1) | ![Graph](image2) | ![Graph](image3) |
| 20 nM | ![Graph](image4) | ![Graph](image5) | ![Graph](image6) |
| 200 nM | ![Graph](image7) | ![Graph](image8) | ![Graph](image9) |
| 10 mM | ![Graph](image10) | ![Graph](image11) | ![Graph](image12) |

B

![Bar Graph](image13)

C

| Control | mRFP | ATP 3 μM |
|---------|------|----------|
| ![Graph](image14) | ![Graph](image15) | ![Graph](image16) |

D

![Bar Graph](image17)

E

| Control | mRFP | TG 1 μM |
|---------|------|---------|
| ![Graph](image18) | ![Graph](image19) | ![Graph](image20) |

F

![Bar Graph](image21)

G

| Control | mRFP | ATP 0.5 μM |
|---------|------|------------|
| ![Graph](image22) | ![Graph](image23) | ![Graph](image24) |

H

![Bar Graph](image25)

I

| Control | mRFP | TG 1 μM |
|---------|------|---------|
| ![Graph](image26) | ![Graph](image27) | ![Graph](image28) |

J

![Bar Graph](image29)
(BZ-8000, Osaka, Japan). Data are presented as mean ± S.D. of at least three independent experiments.

RESULTS

Both GIT1 and GIT2 Bind to All Three IP₃R Subtypes—To understand the function of the CTT of IP₃R (Fig. 1A), we performed a yeast two-hybrid screen of a mixture of embryonic and adult human brain cDNA libraries with the CTT of IP₃R1 as bait to search for novel IP₃R-binding molecules. Nineteen prey clones were obtained. Seven of these clones encoded protein 4.1N fragments (20), and 2 clones encoded the different lengths of the GIT1 fragment (Fig. 1B). The interaction between GIT1 and IP₃R1/CCO was confirmed by a glutathione S-transferase (GST) pull-down assay (Fig. 1C) and also by an immunoprecipitation (IP) assay from mouse whole brain lysates using anti-IP₃R1 (Fig. 1D) and anti-GIT1 antibodies (Fig. 1E). To determine whether both GIT1 and GIT2 bind to all three subtypes of IP₃R, mRFP-fused GIT1 or GIT2 was coexpressed with green fluorescent protein (GFP)-fused IP₃R1, IP₃R2, or IP₃R3, in HeLa cells, and IP was performed using anti-RFP antibody from the cell lysates. All three subtypes of IP₃R were coprecipitated with GIT1 (Fig. 1F) and GIT2 (Fig. 1G), implying that both GIT1 and GIT2 bind to all three subtypes of IP₃R.

The Coiled-coil Domain in the CTT of IP₃R Is Involved in the Interaction with GIT—The interaction between the shorter GIT1 fragment identified from the yeast two-hybrid screen and full-length or serial deletions of the CTT of IP₃R1, IP₃R2, and IP₃R3 were examined by a liquid yeast two-hybrid quantification assay to determine the minimal region responsible for IP₃R binding to GIT. As shown in Fig. 2, A and B, we found that the last 50 (IP₃R1 and IP₃R2) or 44 (IP₃R3) residues within CTT showed 5–8-fold stronger binding to the GIT1 fragment as compared with the full-length CTT of the corresponding IP₃R subtype. Thereafter, we called these peptides “IP₃R/GITCOREs,” which means “GIT-binding cores in IP₃Rs.” Within IP₃R/GITCOREs, the first 10 residues in all three IP₃R/GITCOREs and the last 14 residues in both IP₃R1 and IP₃R2 or the last 8 residues in IP₃R3 are essential for the interaction (Fig. 2, A and B). All three purified GST-fused IP₃R/GITCOREs pulled down the purified GIT1 protein fragment in vitro (Fig. 2, C and D), indicating that the IP₃R/GITCORE is sufficient for GIT binding and that the interaction is direct. By searching functional protein motifs within the CTT of IP₃Rs, we found that a potential coiled-coil domain, which is usually involved in protein-protein interaction, was localized in the CTT of all three IP₃Rs, and that IP₃R/GITCORE partially overlapped with the coiled-coil domain (Fig. 2E). These findings suggest that the coiled-coil domain within the CTT of IP₃R is involved in the GIT/IP₃R interaction.

The Second IQ-like Motif of GIT Is Critical for Binding to IP₃R—To determine the precise IP₃R binding region in GIT, mRFP-fused full-length fragment or subfragments of GIT1 (Fig. 2F) were expressed in HeLa cells and subjected to a pull-down assay using GST-IP₃R2/GITCORE. Peptides containing residues 691–721 of GIT1 bound to IP₃R, whereas peptides not containing these residues failed to bind (Fig. 2G). Because these residues contain the second IQ-like motif, consisting of LQXXR (where X represents any amino acid; Fig. 2, F and I), we further examined whether this IQ-like motif of GIT is involved in the binding. Several single, double, and triple point mutants within this IQ-like motif of mRFP-GIT1 (Fig. 2F) were expressed in HeLa cells and subjected to pull-down assay. We found that mRFP-L712A, L712A/Q713A, and L712A/Q713A/R717A abolished the interaction with GST-IP₃R2/GITCORE (Fig. 2H). Thus, the Leu²¹² residue of GIT1 is critical for binding to IP₃R. By comparing of the homology in the COOH-terminal region of GIT proteins, we found that the Leu²¹² of GIT1 is conserved in all GIT proteins of all species (Fig. 2I), which suggests that the GIT/IP₃R interaction is evolutionarily conserved.

Cytosolic Ca²⁺ Dynamics Modulates the GIT/IP₃R Interaction—Cytosolic Ca²⁺ elevation triggers the conformational change of IP₃R (21). Alternatively, GIT contains putative EF-hand and IQ-like motifs for Ca²⁺ and calmodulin binding, respectively (Fig. 1B). We therefore investigated whether the GIT/IP₃R interaction is regulated by a cytosolic Ca²⁺ concentration by a pull-down assay with GST-IP₃R2/GITCORE against mRFP-GIT1 expressed in HeLa cells in the presence of various concentrations of Ca²⁺. We found that the amount of mRFP-GIT1 pulled down by GST-IP₃R2/GITCORE increased with serial elevation of the Ca²⁺ concentration (Fig. 3A). To corroborate this observation in vivo, HeLa cells were stimulated with 10 μM ATP, an activator of purinergic receptors (Fig. 3B), or 1.0 μM thapsigargin (TG) (Fig. 3C), an inhibitor of the sarcoendoplasmic reticulum Ca²⁺-ATPase, to increase cytosolic Ca²⁺ concentration, and the cell lysates were subjected to the IP assay using anti-GIT1 antibody. We found that both stimulants increased the amount of all three IP₃R subtypes coprecipitated with GIT1. Thus, the above data suggested that an elevated cytosolic Ca²⁺ concentration favors GIT/IP₃R association.

GIT Suppresses IP₃-gated Ca²⁺ Release in Vitro—We investigated whether GIT regulates the channel activity of IP₃R by an in vitro Ca²⁺ release assay using mouse cerebellar microsomes to explore the functional consequences of the GIT/IP₃R interaction.

FIGURE 4. GIT inhibits IP₃-gated Ca²⁺ release. A and B, GIT inhibited in vitro IP₃-gated Ca²⁺ release. Ca²⁺ release from mice cerebellar microsome incubated with or without recombinant wild-type (His-GIT) or mutant (His-L712A) GIT1 was measured using fura-2, a Ca²⁺ indicator, and addition of the indicated concentrations of IP₃, A, representative time courses of Ca²⁺ release. IP₃ was added at the times indicated by the arrowheads. B, average peak heights of IP₃-induced Ca²⁺ release. Ca²⁺ release was normalized to total releasable Ca²⁺ (0.1% Triton X-100, p < 0.05; t test; compared with control and His-L712A). Data are presented as mean ± S.D. of at least three independent experiments. C–J, overexpression of GIT inhibited IP₃-gated Ca²⁺ release in HeLa and COS-7 cells. Cells expressing mRFP, mRFP-GIT1, or mRFP-L712A were stimulated with the indicated concentrations of ATP or TG in extracellular Ca²⁺-free medium and Ca²⁺ release was recorded by Ca²⁺-imaging experiments. For quantitation, normalized responses were calculated with averaged Ca²⁺ response (AUC) of non-expressing cells, set equal to 100%. C, representative ATP-stimulated Ca²⁺ responses in HeLa cells. D, quantitation of the results in C. E, representative TG-stimulated Ca²⁺ responses in HeLa cells. F, quantitation of the results in E. G, representative ATP-stimulated Ca²⁺ responses in COS-7 cells. H, quantitation of the results in G. I, representative TG-stimulated Ca²⁺ responses in COS-7 cells. J, quantitation of the results in I. *p < 0.05; **p < 0.005; ***p < 0.005 (t test; compared with mRFP and mRFP-L712A). Data are presented as mean ± S.D. of at least five independent experiments.
action. The microsomes were preincubated with or without recombinant wild-type or mutant GIT1, and the Ca\(^{2+}\) release activity was examined by addition of various concentrations of IP\(_3\). We found that wild-type GIT1 reduced Ca\(^{2+}\) release in response to low concentrations of IP\(_3\) (2–200 nM), which were in the range of physiological concentrations (22), whereas the L712A point mutant GIT1 markedly attenuated the ability to suppress Ca\(^{2+}\) release (Figs. 4, A and B). These results suggest that the GIT–IP\(_3\)R interaction suppresses the channel activity of IP\(_3\)R by reducing the apparent sensitivity of the channel to IP\(_3\).

**GIT Inhibits IP\(_3\)-gated Ca\(^{2+}\) Release in Intact Cells**—To investigate whether GIT also modulates IP\(_3\)-gated Ca\(^{2+}\) release in intact cells, we imaged Ca\(^{2+}\) signals of HeLa cells expressing mRFP-fused wild-type or mutant GIT1, or mRFP alone in response to 3.0 μM ATP stimulation in an extracellular Ca\(^{2+}\)-free medium. Whereas the Ca\(^{2+}\) response of cells expressing mRFP or mRFP-L712A was indistinguishable from that of non-expressing cells, cells expressing mRFP-GIT1 typically exhibited significantly smaller Ca\(^{2+}\) transients (Fig. 4, C and D). The difference in Ca\(^{2+}\) transients in cells expressing mRFP-GIT1 was not due to a decrease in Ca\(^{2+}\) store size within the ER because the amount of passive Ca\(^{2+}\) leakage elicited by TG was unchanged (Fig. 4, E and F). We also observed a similar effect of GIT1 overexpression on IP\(_3\)-induced Ca\(^{2+}\) release in COS-7 cells (Fig. 4, G–J). Hence, we conclude that GIT generally inhibits the IP\(_3\)-gated Ca\(^{2+}\) release in intact cells.

**Specific Knockdown of GIT Results in Amplified Intracellular Ca\(^{2+}\) Oscillations and IP\(_3\)R Channel Activity**—Next, we used the RNA interference technique to examine the effect of endogenous GIT on IP\(_3\)R channel activity. Because there are no suitable antibodies that specifically recognize GIT2 because of the high homology between GIT1 and GIT2 (13), we used a pan-GIT antibody that almost equally recognizes GIT1 and GIT2 to examine antibodies that specifically recognize GIT2 because of the difference in Ca\(^{2+}\) response as AUC. *, p < 0.05; **, p < 0.01; ***, p < 0.005 (t test; compared with control siRNA). Data are presented as mean ± S.D. of at least five independent experiments.

**GIT Knockdown Induces Caspase-dependent Apoptosis**—We noticed that many cells transfected with GIT siRNAs died, and therefore we examined whether apoptosis occurred in these cells. The GIT siRNA-transfected HeLa cells showed a significantly increased number of TUNEL-positive cells as compared with non-siRNA and control siRNA-transfected cells (Fig. 6, A and C). The increased number of TUNEL-positive cells was abolished by preincubation with Z-VAD-fmk, a pan-inhibitor for caspases (Fig. 6, B and C), confirming the involvement of caspase activation in GIT1 siRNA-transfected cells. Cleaved caspase-9 and caspase-3 were consistently detected only in cells transfected with GIT1 siRNA (Fig. 6D, lane 3); Z-VAD-fmk efficiently blocked the activation of these two caspases (Fig. 6D, lane 6).

**GIT Knockdown Accelerates Staurosporine (STS)-stimulated Apoptosis**—It is known that apoptosis-inducible stimuli, such as STS (23–25) and ceramide (26), can induce IP\(_3\)R Ca\(^{2+}\) release. We therefore examined the effect of GIT knockdown on STS-stimulated apoptosis to further investigate the role of GIT in apoptosis. Forty-eight hours after transfection with GIT1 siRNA, we treated the HeLa cells with 1 μM STS for 4 h and examined the number of apoptotic cells. We observed a significant increase in TUNEL-positive cells among the GIT1 siRNA-transfected cells (Fig. 6, E and G). STS-stimulated apoptosis in the GIT1 siRNA-transfected cells was abolished by Z-VAD-fmk treatment (Fig. 6, F and G). In addition, Western blot analysis revealed the activation of caspase-9 and caspase-3 only in GIT1 siRNA-transfected cells that were treated with STS for 4 h (Fig. 6H, lane 9). Although the activation of caspase-9 and caspase-3 was detected even in control cells after STS treatment for 8 h (Fig. 6H, lanes 13–15), it was much stronger in cells transfected with GIT1 siRNA (Fig. 6H, lane 15).

![Figure 5](image-url) **Figure 5. Knockdown of GIT amplified IP\(_3\)-gated Ca\(^{2+}\) oscillations and Ca\(^{2+}\) release.** A–C, knockdown of GIT in HeLa and COS-7 cells. A, specificities of two antibodies for GIT. B and C, the expression of GIT1 and GIT2 in HeLa (B) and COS-7 cells (C). D–H, the effects of knockdown of GIT on IP\(_3\)-gated Ca\(^{2+}\) oscillations and Ca\(^{2+}\) release in HeLa cells. D, representative Ca\(^{2+}\) response. E–G, percentages of the cells with discernible Ca\(^{2+}\) response (E) and of the cells that responded with oscillations (F) or spikes (G) at the indicated ATP concentrations; response, Ca\(^{2+}\) peaks > 0.05 μM, oscillation, at least three Ca\(^{2+}\) peaks > 0.05 μM, spike, one or two Ca\(^{2+}\) peaks < 0.05 μM. H, quantitation of Ca\(^{2+}\) response as AUC. I–M, the effects of knockdown of GIT1 and/or GIT2 on IP\(_3\)-gated Ca\(^{2+}\) oscillations and Ca\(^{2+}\) release in COS-7 cells. Ca\(^{2+}\) responses were analyzed as in E–H. I, representative Ca\(^{2+}\) response. J–L, percentages of the cells with discernible Ca\(^{2+}\) response (J) and the cells responded with oscillations (K) or spikes (L) at the indicated ATP concentrations. M, quantitation of Ca\(^{2+}\) response as AUC. *, p < 0.05; **, p < 0.01; ***, p < 0.005 (t test; compared with control siRNA). Data are presented as mean ± S.D. of at least five independent experiments.
FIGURE 6. Knock-down of GIT-induced and accelerated caspase-dependent apoptosis. A–D, knockdown of GIT1-induced caspase-dependent apoptosis. Twenty-four hours after transfection without (No siRNA) or with control or GIT1 siRNA, HeLa cells were incubated without or with 50 \( \mu \)M Z-VAD-fmk. After another 72 h, the cells were processed for TUNEL staining or Western blot. A and B, representative TUNEL coupled with 4',6-diamidino-2-phenylindole (DAPI) staining of cells in the absence (A) or presence (B) of Z-VAD-fmk. C, percentages of TUNEL-positive cells in A and B. D, representative Western blot results. E–H, knockdown of GIT1 accelerated STS-stimulated apoptosis. Forty-eight hours after transfection without (No siRNA) or with control or GIT1 siRNA, HeLa cells were treated with or without 1 \( \mu \)M STS for the indicated times in the absence or presence of 50 \( \mu \)M Z-VAD-fmk. Cells were analyzed as in A–D. E and F, representative TUNEL coupled with 4',6-diamidino-2-phenylindole staining of cells treated with STS for 4 h in the absence (E) or presence (F) of Z-VAD-fmk. G, percentages of TUNEL-positive cells in E and F. H, representative Western blot results of cells treated without (0 h) or with STS for 4 and 8 h in the absence or presence of Z-VAD-fmk. Scale bar, 100 \( \mu \)M. ****, \( p < 0.0001 \) (t test; compared with control siRNA). Data are presented as mean ± S.D. of at least four independent experiments.
Activated caspase-9 and caspase-3 were not detected when the cells were pretreated with Z-VAD-fmk (Fig. 6H, lanes 12 and 16–18). Altogether, these data demonstrate that GIT has a critical role in protection against caspase-dependent apoptosis.

**GIT Inhibits STS-stimulated Apoptosis by Interacting with IP₃R**—We then examined the effect of overexpression of the wild-type or L712A-mutant GIT on apoptosis in GIT1 siRNA-transfected HeLa cells to verify whether GIT inhibits apoptosis by modulating IP₃R channel activity. We found that the overexpression of mRFP-GIT1 attenuated the acceleration of apoptosis in GIT1 siRNA-transfected cells induced by STS treatment for both 4 (Fig. 7A, lane 11) and 8 h (Fig. 7A, lane 17). In contrast, overexpression of mRFP-L712A abolished the attenuating effect: apoptosis observed in cells expressing mRFP-L712A (Fig. 7A, lanes 12 and 18) was similar in cells expressing mRFP alone (Fig. 7A, lanes 10 and 16). We also found that apoptosis in the control HeLa cells, triggered by 8 h STS treatment, was attenuated by the wild-type and not the mutant form of GIT1 (Fig. 7A, lanes 13–15). Furthermore, application of 2-aminoethoxydiphenyl borate (Fig. 7B, lane 4) and xestospongin C (Fig. 7B, lane 5), two pharmacological inhibitors of IP₃R, inhibited the acceleration of apoptosis in GIT1 siRNA-transfected cells. Altogether, we conclude that the inhibitory effect of GIT on caspase-dependent apoptosis is caused by the modulation of cytosolic Ca²⁺ concentration and produces cytosolic Ca²⁺ oscillations, which may activate the caspase cascade. The elevated cytosolic Ca²⁺ concentration simultaneously enhances GIT-IP₃R interaction, thereby attenuating Ca²⁺ release and cytosolic Ca²⁺ oscillations and inhibiting the activation of the caspase cascade.

**DISCUSSION**

Inhibition of Apoptosis by GIT Regulation of IP₃R-mediated Ca²⁺ Signal—Here, we found that GIT functions as an anti-apoptotic modulator by inhibiting IP₃R activity. Based on the following two observations, we conclude that GIT presumably inhibits channel activity by elevating the threshold of the IP₃ concentration necessary for gating of the channel. First, purified GIT1 reduced IP₃R channel activity in vitro in response to a low concentration of IP₃ (Fig. 4, A and B). Second, knockdown of both GIT proteins resulted in more cells responding to a lower but not to a higher concentration of the IP₃-generating agonist without a change in the level of IP₃ production (Fig. 5, D–M; data not shown). Based on these data, we propose a model by which GIT regulates apoptosis through IP₃R (Fig. 7, C–E). In resting cells, in which the cytosolic Ca²⁺ concentration is low (∼10⁻⁷ M), relatively small amounts of GIT bind to the IP₃R channel, and the channel activity is high. Under STS treatment, Ca²⁺ entry through the L-type Ca²⁺ channel enhances the Ca²⁺ concentration and produces cytosolic Ca²⁺ oscillations, which may activate the caspase cascade. The elevated cytosolic Ca²⁺ concentration simultaneously enhances GIT-IP₃R interaction, thereby attenuating Ca²⁺ release and cytosolic Ca²⁺ oscillations and inhibiting the activation of the caspase cascade.
**GIT Proteins Inhibit Apoptosis**

IP₃R with low affinity and desensitize IP₃R to IP₃ that may exist in unstimulated cells, thereby inhibiting the opening of IP₃Rs (Fig. 7C). When the cells are stimulated and the IP₃ concentration rises, IP₃R binds to IP₃ and releases Ca²⁺ from the ER (Fig. 7D). Subsequently, the elevated cytosolic Ca²⁺ concentration enhances GIT-IP₃R association, which in turn inhibits IP₃R channel activity as a negative feedback (Fig. 7E), thereby inhibiting the elevation of excessive cytosolic Ca²⁺ and preventing the activation of the caspase cascade. Interestingly, several key regulators of apoptotic signaling, such as cytochrome c (9), Bcl-2 (10), and Bcl-xl (12), have been reported to interact with the CTT of IP₃R (Rong et al. (11) argued that Bcl-2 interacts with the internal coupling domain, but not the CTT of IP₃R) and modulate IP₃R activity by changing the apparent affinity for IP₃. Together with previous findings that cysteine mutation within the CTT (3) or deletion of the coiled-coil domain from the CTT (27) impairs channel activity, the CTT of IP₃R, by itself and with accessory proteins, plays a critical role in regulating IP₃R channel opening. Because the CTT is reported to contribute to IP₃R tetramer stability (27), it would be interesting to study the relationship between the effect of accessory proteins on structural changes of IP₃R and the channel activity.

Why does GIT knockdown increase the number of cells showing Ca²⁺ oscillations in cells (Fig. 5, D, F, I, and K)? One possible explanation is the affinity of GIT for each IP₃R subtype. Previous studies have found that each IP₃R subtype has different biophysical properties; for example, IP₃R1 and IP₃R2 tend to generate Ca²⁺ oscillations, whereas IP₃R3 acts as an anti-Ca²⁺ oscillatory unit in HeLa and COS-7 cells (28). IP₃R2 is required for long-lasting regular Ca²⁺ oscillations, whereas IP₃R3 generates only monophasic Ca²⁺ responses in DT40 cells (29). Kuroda et al. (30) recently demonstrated that IP₃R2 contributes to Ca²⁺ oscillation in osteoclasts, using IP₃R knock-out mice. Although we should regard these results with caution because of the presence of so many regulatory factors of IP₃R activity in vivo (for example, Ca²⁺, IP₃, ATP, accessory proteins, and IP₃R localization), these papers suggest that both IP₃R2 and IP₃R1, particularly IP₃R2, generally contribute to the generation of Ca²⁺ oscillations in vivo. Judging from our data showing that GIT seems to bind to IP₃R2 and IP₃R1 with higher affinity compared with IP₃R3 in vivo (Figs. 1, F and G, and 2A), GIT may predominantly inhibit IP₃R1 and IP₃R2 that contribute to the generation of Ca²⁺ oscillations in HeLa and COS-7 cells. Thus, GIT knockdown would increase the number of cells showing Ca²⁺ oscillations.

**Regulation of the GIT-IP₃R Interaction by Cytosolic Ca²⁺ Dynamics**—The precise mechanism by which Ca²⁺ enhances the GIT-IP₃R interaction (Fig. 3) is still unknown, although conformational changes in IP₃R and/or GIT may underlie this Ca²⁺-dependent interaction. It was demonstrated that the NH₂-terminal IP₃ binding region, which was shown to bind to the S4–S5 linker near the CTT (27), relocates from the corners of the square form to the corners of the radial wings of the windmill form in the presence of Ca²⁺ (21). This Ca²⁺-dependent spatial rearrangement of the NH₂- and COOH-terminal regions of IP₃R may at least in part facilitate the accessibility of GIT to the CTT of IP₃R. Alternatively, Ca²⁺ may change the conformation of GIT. Because GIT has an IQ-like motif critical for IP₃ binding (Fig. 2, F–I) and some interactions between proteins containing IQ-like motifs and their partners can be regulated by Ca²⁺ (31), the putative Ca²⁺-binding EF-hand of GIT (Fig. 1B) might be responsible for the Ca²⁺-dependent GIT-IP₃R interaction. Further studies on the structural and biochemical regulation of GIT and IP₃R by Ca²⁺ will be necessary to elucidate the precise mode of their Ca²⁺-dependent interaction.

**Significance of the Identification of GIT as an Anti-apoptotic Molecule**—Our data will shed light on the diverse physiological and pathological significance of GIT-dependent apoptotic processes in the future because apoptosis is not only essential for normal tissue development and homeostasis but also contributes to many forms of pathological cell loss. For example, the COOH-terminal fragment of GIT1, which was shown to bind to IP₃R in this study, has been found in Huntington disease but not in the healthy brain (32) and decreases the number of synapses and neurite formation (33, 34). Nef, a protein that is encoded by the human immunodeficiency virus (HIV)-1 and HIV-2, which can induce apoptosis in the cells of the immune system (35), has been reported to associate with GIT in HIV-1-infected primary T lymphocytes and macrophages (36), and with IP₃R1 in Nef-transfected Jurkat T cells and HIV1-infected primary human peripheral mononuclear cells (37). It would be interesting to investigate the possible association of the full-length and/or COOH-terminal fragment of GIT with IP₃R and its effect on neurogenesis, neurodegeneration, and HIV infection, an endeavor that might yield new diagnostic or therapeutic tools.

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**REFERENCES**

1. Berridge, M. J. (1993) *Nature* 361, 315–325
2. Mikoshiba, K. (2007) *J. Neurochem.* 102, 1426–1446
3. Uchida, K., Miyachi, H., Furuichi, T., Michikawa, T., and Mikoshiba, K. (2003) *J. Biol. Chem.* 278, 16551–16560
4. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) *Physiol. Rev.* 87, 593–658
5. Orrenius, S., Zhivotovsky, B., and Nicotera, P. (2003) *Nat. Rev. Mol. Cell Biol.* 4, 552–565
6. Joseph, S. K., and Hajnoczky, G. (2007) *Apoptosis* 12, 951–968
7. Nagata, S. (2000) *Exp. Cell Res.* 256, 12–18
8. Goll, D. E., Thompson, V. F., Li, H., Wei, W., and Cong, J. (2003) *Physiol. Rev.* 83, 731–801
9. Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosi, T., and Snyder, S. H. (2003) *Nat. Cell Biol.* 5, 1051–1061
10. Chen, R., Valencia, I., Zhong, F., McColl, K. S., Roderick, H. L., Bootman, M. D., Berridge, M. J., Conway, S. J., Holmes, A. B., Mignery, G. A., Velez, P., and Distelhorst, C. W. (2004) *J. Cell Biol.* 166, 193–203
11. Rong, Y. P., Aromolaran, A. S., Bultynck, G., Zhong, F., Li, X., McColl, K., Matsuyama, S., Herlitze, S., Roderick, H. L., Bootman, M. D., Mignery, G. A., Parys, J. B., De Smedt, H., and Distelhorst, C. W. (2008) *Mol. Cell Biol.* 31, 255–265
12. White, C., Li, C., Yang, J., Petenko, N. B., Madesh, M., Thompson, C. B., and Foskett, J. K. (2005) *Nat. Cell Biol.* 7, 1021–1028
13. Hoefen, R. J., and Berk, B. C. (2006) J. Cell Sci. 119, 1469–1475
14. Nakayama, T., Hattori, M., Uchida, K., Nakamura, T., Tateishi, Y., Bannai, H., Iwai, M., Michikawa, T., Inoue, T., and Mikoshiba, K. (2004) Biochem. J. 377, 299–307
15. Iwai, M., Tateishi, Y., Hattori, M., Mizutani, A., Nakamura, T., Futatsugi, A., Inoue, T., Furuchi, T., Michikawa, T., and Mikoshiba, K. (2005) J. Biol. Chem. 280, 10305–10317
16. Zhang, S., Malmersjö, S., Li, J., Ando, H., Aizman, O., Uhlen, P., Mikoshiba, K., and Aperia, A. (2006) J. Biol. Chem. 281, 21954–21962
17. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
18. Sugiyama, T., Yamamoto-Hino, M., Miyawaki, A., Furuchi, T., Mikoshiba, K., and Hasegawa, M. (1994) FEBS Lett. 349, 191–196
19. Ando, H., Mizutani, A., Kiefer, H., Tsuzurugi, D., Michikawa, T., and Mikoshiba, K. (2006) Mol. Cell 22, 795–806
20. Zhang, S., Mizutani, A., Hisatsune, C., Higo, T., Bannai, H., Nakayama, T., Hattori, M., and Mikoshiba, K. (2003) J. Biol. Chem. 278, 4048–4056
21. Hamada, K., Miyata, T., Mayanagi, K., Hirota, J., and Mikoshiba, K. (2002) J. Biol. Chem. 277, 21115–21118
22. Luzzi, V., Sims, C. E., Soughayer, J. S., and Allbritton, N. L. (1998) EMBO J. 17, 3065–3072
23. Szalai, G., Krishnamurthy, R., and Hajnóczky, G. (1999) EMBO J. 18, 6349–6361
24. Nutt, L. K., Chandra, J., Pataer, A., Fang, B., Roth, J. A., Swisher, S. G., O’Neil, R. G., and McConkey, D. J. (2002) J. Biol. Chem. 277, 20301–20308
25. Asefa, Z., Bultynck, G., Szulcik, K., Nadif Kasri, N., Vermassen, E., Goris, J., Missiaen, L., Callewaert, G., Parys, J. B., and De Smedt, H. (2004) J. Biol. Chem. 279, 43227–43236
26. Pinto, P., Ferrari, D., Rapizzi, E., Di Virgilio, F., Pozzan, T., and Rizzuto, R. (2001) EMBO J. 20, 2690–2701
27. Schug, Z. T., and Joseph, S. K. (2006) J. Biol. Chem. 281, 24431–24440
28. Hattori, M., Suzuki, A. Z., Higo, T., Miyawaki, M., Michikawa, T., Nakamura, T., Inoue, T., and Mikoshiba, K. (2004) J. Biol. Chem. 279, 11967–11975
29. Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Iino, M. (1999) EMBO J. 18, 1303–1308
30. Kuroda, Y., Hisatsune, C., Nakamura, T., Matsuo, K., and Mikoshiba, K. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 8643–8648
31. Bähler, M., and Rhoads, A. (2002) FEBS Lett. 513, 107–113
32. Goehler, H., Lalowski, M., Stelzl, U., Waelder, S., Stroedicke, M., Worm, U., Droge, A., Lindenberg, K. S., Knoblich, M., Haenig, C., Herbst, M., Suopanki, J., Scherzinger, E., Abraham, C., Bauer, B., Hasenbank, R., Fritzschke, A., Luderig, A. H., Büsow, K., Coleman, S. H., Gutekunst, C. A., Landwehrmeyer, B. G., Lehrh, H., and Wanker, E. E. (2004) Mol. Cell 15, 853–865