Novel small GTPase subfamily capable of associating with tubulin is required for chromosome segregation

Takuro Okai, Yasuhiro Araki, Minoru Tada, Toshiyuki Tateno, Kenji Kontani and Toshiaki Katada*

Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.

*Author for correspondence (e-mail: katada@mol.f.u-tokyo.ac.jp)

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Summary

The small GTPase superfamily, which includes the Ras, Rho/Rac, Rab, Arf and Ran subfamilies, serves as a signal transducer to regulate cell proliferation and differentiation, actin cytoskeleton, membrane trafficking, and nuclear transport. Here, we identify novel GTPases (human Gie1 and Gie2) that form a distinct subfamily of the small GTPases in terms of their sequences and intracellular function. Gie stands for 'novel GTPase indispensable for equal segregation of chromosomes', and this subfamily is conserved in multicellular organisms. Expression of dominant-negative Gie mutants in mammalian cells or knockdown of Gie transcripts using RNA interference in *Drosophila* S2 cells induced abnormal morphology in the chromosome segregation. Gie protein has ability to bind to tubulin and localizes with microtubules on the spindle mid-zone in late mitosis. Furthermore, overexpression of Gie mutants that lack putative effector domains but have tubulin-binding ability induced micronucleus formation. Thus, this is the first report showing that a small GTPase subfamily capable of associating with microtubules might be involved in chromosome segregation.

Key words: Chromosome segregation, Mitosis, Small GTPase, Tubulin

Introduction

The superfamily of small GTPases serves as a signal transducer to regulate a diverse array of cellular functions. The members of this superfamily are structurally and functionally classified into at least five groups – Ras, Rho/Rac, Rab, Arf and Ran – and they are involved in the control of cell proliferation and differentiation, regulation of the actin cytoskeleton, membrane trafficking, and nuclear transport (Campbell et al., 1998; Hall, 1998; Moore, 1998; Moss and Vaughan, 1998; Zerial and McBride, 2001). All the GTPases contain five highly conserved GTP-binding domains, termed G1-G5, and function as molecular switches in a manner dependent on their guanine-nucleotide-bound forms (Takai et al., 2001). For example, the prototypic Ras proteins transduce signals for cell growth and differentiation by cycling between GTP-bound active and GDP-bound inactive states (Bourne et al., 1991). The activation mechanism of small GTPases typically involves GDP-GTP exchange reaction that is stimulated by guanine-nucleotide exchange factors (GEFs), and GTP binding triggers an allosteric movement of their switch 1 and switch 2 regions to facilitate effector interaction. In the GTP-bound active state, small GTPases interact through their effector domains with a range of cellular targets to elicit their biological functions. By contrast, the turn-off mechanism involves GTPase activity intrinsically present in this family, and GTPase-activating proteins (GAPs) stimulate the GTP-hydrolysis reaction.

At present, approximately 150 members of this superfamily have been identified in humans (Heo and Meyer, 2003), some of which are characterized by the presence of unique amino-acid sequences distinguishable from the well-known small GTPase subfamily (Antoshechkin and Han, 2002; Bhamidipati et al., 2000; Ellis et al., 2002; Kontani et al., 2002; Piddini et al., 2001). Identification of these atypical members has expanded our understanding of the roles of small GTPases in cell biology and they are likely to serve as distinct regulators of uncharacterized signaling cascades. It is thus expected that further studies of other subfamilies of the small GTPases would also reveal novel signaling pathways involved in a range of cell functions.

During cell division, the two daughter cells must inherit the same genetic background, and errors in this process cause birth defects and contribute to tumor progression. Cell division consists of mitosis and cytokinesis (for reviews, see Glotzer, 2001; Guertin et al., 2002; Scholey et al., 2003). Mitosis, which is the creation of genetically identical cells from a single cell, involves the segregation of chromosomes to daughter cells via a microtubule-based structure known as the mitotic spindle. Although the mechanical details of mitosis are known, the molecular mechanisms of chromosome segregation are poorly understood.

Several members of the small GTPases are known to be involved in cell division. The Rho family is most prominent in carrying out essential functions of cytokinesis (Hall, 1998). Inactivation of Rho in animal cells inhibits cytokinesis by disrupting the normal assembly of actin filaments and triggering disassembly of the contractile ring. Rho localizes to the cleavage furrow and mid-body during cytokinesis. Regulators of Rho have also been shown to play important roles in cytokinesis. A Rho-GEF (human ECT2 and its *Drosophila* homolog pebble) localizes to the spindle mid-zone

during cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). A Rho-GAP (CYK-4) identified in *Caenorhabditis elegans* also localizes to the spindle mid-zone (Jantsch-Plunger et al., 2000). CYK-4 and the kinesin-like protein ZEN-4 show a mutual dependence for localization, suggesting that the two spindle mid-zone proteins might co-operate in executing cytokinesis. In addition, Ran has been demonstrated to regulate microtubule polymerization in a manner independent of its role in nuclear transport (Dasso, 2002). During mitosis, the importin-β import receptor acts with its heterodimeric partner, importin-α, to bind and inhibit factors required for spindle assembly. Targets of this inhibition include the mitotic spindle proteins TPX2 and NuMA. A locally high concentration of GTP-bound Ran generated by RCC1 might release the inhibition by importin-β near chromosomes and thereby facilitate bipolar spindle formation. Moreover, Ran acts as a molecular switch to silence spindle-checkpoint signals (Arnaoutov and Dasso, 2003). GTP-Ran releases checkpoint proteins including Bub1, Bub3, Mad2 and CENP-E from kinetochores, which activates APC and allows the transition from metaphase to anaphase. Recently, Cdc42 and its effector mDia3 were shown to regulate microtubule attachment to kinetochores for proper chromosome alignment and segregation (Yasuda et al., 2004).

In the present study, we identified novel members of the small GTPases, termed Gie1 and Gie2, which stands for 'GTPases indispensable for equal segregation of chromosomes'. Reducing Gie activity by either overexpression of dominant-negative Gie mutants or RNA interference (RNAi) induces abnormal morphology in the chromosome segregation. We also show that Gie is capable of associating with microtubules in both in vitro and living cells. Thus, Gie appears to play an essential role in chromosome segregation.

Materials and Methods

cDNA cloning of Gie and database analyses

Database searches using Entrez (NCBI's search and retrieval system) were performed to find out novel small GTPases. Sequence data from the open reading frame of the cosmids (accession numbers AK001564 and BC015408) were used to design specific primers to amplify the cDNA fragments of Gie1 and Gie2. Primers used were: Gie1 (5′-ATGCTGGCGCTCATCTCC-3′ and 5′-TCAGCTTCTTCTAGATTTTGAATGCTG-3′) and Gie2 (5′-ATGATCGCTTTGTTCAACAAGCTG-3′ and 5′-TCAGCTTCTCCGTGACTTCGA-3′). Polymerase chain reaction (PCR) amplification was performed using human whole-brain cDNA (Clontech) and KOD polymerase (TOYOBO) in 30 cycles (98°C for 15 seconds, 65°C for 2 seconds and 74°C for 30 seconds). PCR products thus obtained were sequenced. DNA-sequencing reactions were performed using the DYEnamic ET Terminator Cycle Sequencing Premix Kit (Amersham Pharmacia Biotech). Samples were analysed using an ABI 373 DNA Sequencer and ABI Prism Model version 2.1.1 software (PE Applied Biosystems). Alignment of Gie proteins to related protein sequences was carried out using ClustalW and viewed in GeneDoc. An unrooted phylogenetic tree was prepared using ClustalW and viewed in TreeView (Thompson et al., 1994).

Northern blot analysis

Expression patterns of *hGie1* and *hGie2* mRNAs were analysed using human multiple-tissue northern blot (Clontech). The labeled probes for Gie1 and Gie2 were prepared using the cDNAs of the coding sequences of *hGie1* and *hGie2* (561 bp each) and Rediprime II Random Prime Labelling System (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Hybridization was carried out in the ExpressHyb hybridization solution (Clontech) in the presence of ^{32}P-cDNA probes. The membrane was washed twice with 2× SSC (1× SSC consists of 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS, twice with 0.5× SSC and 0.1% sodium dodecyl sulfate (SDS), and once with 0.2× SSC and 0.1% SDS at 68°C. Filters were exposed to X-ray film (FujiFilm) at –80°C for 2-3 days with an intensifying screen.

Production of anti-Gie antibody and immunoblotting

To generate an anti-Gie antibody, the C-terminal peptide (CLIQHSKSRRS) of human Gie1 and Gie2 was conjugated with keyhole limpet hemocyanin and injected into rabbits. The antibody was purified from whole serum with the peptide-immobilized affinity column (Pierce). Immunoblotting was performed as described previously (Saito et al., 2002). The reagents used were anti-β-tubulin polyclonal antibody (Santa Cruz Biotechnology) and anti-glyceraldehyde-3-phosphate-dehydrogenase (anti-GAPDH) antibody (Chemicon).

Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 0.16% (w/v) $NaHCO_3$, 0.6 mg ml^{-1} L-glutamine, 100 μg ml^{-1} streptomycin, 100 IU ml^{-1} penicillin at 37°C in 95% air, 5% CO_2. PC12 cells were maintained in the above DMEM further supplemented with 5% horse serum. S2 cells were grown in 1× Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FCS, 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin in a 75-cm^2 T-flask (Sarstedt) at room temperature.

HeLa cells were transfected with 1 μg (35 mm dish) or 3 μg (60 mm dish) of plasmid DNA using LipofectAMINE 2000 (Invitrogen). For electroporation, the cells (1×10^7 cells) were washed twice and resuspended in 0.2 ml Opti-MEM (Gibco). The cell suspension was mixed with 10 μg of plasmids and transferred to a 0.4-cm-gap electroporation cuvette (BioRad). After being electroporated (220 V, 960 μF), the cells were diluted into 20 ml DMEM and cultured at 37°C for 2-3 days.

Radiolabeling of nucleotides associated with small GTPases and identification of the nucleotide-bound forms

Gie- and Ha-Ras-encoding sequences were inserted into pFLAG-CMV5 vector, and the FLAG-tagged proteins were expressed in HeLa cells. The expression level was confirmed by immunoblot analysis with an anti-FLAG monoclonal antibody (Sigma). Guanine nucleotides associated with the GTP-binding proteins were analysed essentially as described previously (Muroya et al., 1992). Briefly, the cells that had been cultured in 60 mm dishes for 24 hours after the transfection were labeled with ^{32}P (1.85 MBq per dish) in phosphate-free DMEM for 4 hours. The labeled cells (3×10^6 cells) were lysed with 1 ml of an ice-cold solubilizing buffer [40 mM Na-HEPES (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol (DTT), 1% (w/v) NP-40, 2 μg ml^{-1} aprotinin, 0.5 mM Pefabloc SC (Roche)] and clarified. The precleared lysates were incubated with anti-FLAG-antibody-conjugated beads (M2-Agarose-Affinity; Sigma) at 4°C for 2 hours. After extensive washing of the immunocomplexes, associated nucleotides were separated by thin-layer chromatography and quantified with a BAS-1800 image analyzer (FujiFilm).

Immunofluorescence study and microscopic observation

Immunofluorescence study was performed as described previously

(Kajiho et al., 2003). Briefly, HeLa cells transiently expressing FLAG-tagged proteins and PC12 cells were cultured on a poly-L-lysine-coated cover glass (15 mm diameter) and washed three times with PBS, followed by fixation with 4% paraformaldehyde in PBS at 25°C for 15 minutes. After permeabilization with 0.02% Triton X-100 in PBS for 10 minutes, the cells were incubated with a blocking solution [5% bovine serum albumin (BSA) in TBS] for 30 minutes, followed by incubation with the indicated antibodies (1 μg ml^{-1} diluted with 5% BSA in TBS) at 37°C for 2 hours. The cells were washed three times with PBS and incubated for 1 hour with Alexa-488- or Alexa-568-conjugated secondary antibodies (Molecular Probes) diluted with the blocking solution. After washed three times with PBS, the coverglass was mounted onto a glass slide in Permafluor mounting medium (Immunon) and viewed on a Carl Zeiss confocal microscope with LSM510 software using excitation wavelengths of 488 nm or 546 nm. The images were merged using Adobe Photoshop (Adobe Systems, Mountain View, CA). The reagents used were anti-α-tubulin monoclonal antibody (Molecular Probes), 4′,6-diamidino-2′-phenylindole (DAPI) (Molecular Probes) and PicoGreen (Molecular Probes). S2 cells were fixed and permeabilized according to the methods described previously (Rogers et al., 2002). Then cells were blocked with 5% BSA in TBS and stained with indicated antibodies as described above.

Double-stranded-RNA preparation and transfection

Drosophila Gie expressed sequence tag (EST) clone (clone ID SD26145, accession number BI632379) was purchased from Invitrogen. A DNA fragment containing the coding sequence was amplified by using PCR. Each primer used in the PCR contained a 5′-T7 RNA-polymerase-binding site (GAATAATACGACTCAC-TATAGGGAGA) followed by sequences specific for the targeted genes. The PCR product was used as a template using the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX) to produce double-stranded RNA (dsRNA). The dsRNA was annealed by incubation at 75°C for 5 minutes and slowly cooled down to room temperature (1°C per minute). The dsRNA product was precipitated with 2-propanol and resuspended in water. Primer sequences used to generate a specific dsRNA were dGie (5′-ATGTTGGCCCTCAT-CAACAGGA-3′ and 5′-CTAACGACTTTGGCTTTTCGAATGT-3′) and β-galactosidase coded in pBluescript II (Promega) (5′-CACT-CAACCCTATCTCGGTC-3′ and 5′-CATGTTCTTTCCTGCGT-TATCCC-3′).

RNAi was carried out by the method of Clemens (Clemens et al., 2000) with slight modification. *Drosophila* S2 cells were diluted to a final concentration of 1×10^6 cells ml^{-1} in a serum-free medium (*Drosophila* expression system; Invitrogen). One 1-ml aliquot per well was plated on a six-well culture dish (Corning) and dsRNA (15 μg of approximately 700 bp) was added directly to the medium with vigorous agitation. The cells were incubated at room temperature for 30 minutes followed by adding 2 ml of 1× Schneider's medium containing 10% FBS. The cells were incubated for additional 4 days to allow for turnover of the target protein. β-Galactosidase was used as a negative control.

Semi-quantitative PCR analysis

To assess the efficacy of RNAi, total RNA of the dsRNA-treated S2 cells were isolated with Trizol reagent (Invitrogen) and first-stranded cDNA was synthesized by using SuperScript™ II RNase H^- reverse transcriptase (Invitrogen). Semi-quantitative PCR analysis was performed using a fourfold dilution series of input cDNA as described previously (Anderson et al., 2002). The following paired primers used as a control were: dAurora B (5′-ATGACGCTTTCCCGCGC-3′ and 5′-TCAATTCCTGGCCGTGTTCTC-3′). PCR reactions were carried out in a final volume of 20 μl, using 0.05 μl of recombinant Taq DNA polymerase and 500 nM of each primer in 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl). Cycling conditions were a single denaturing step at 94°C for 2 minutes followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

Flow-cytometry analysis

S2 cells were washed with ice-cold PBS, fixed with 70% (v/v) ethanol and treated with 1 mg ml^{-1} RNase A at 37°C for 30 minutes. The cells were stained with 50 μg ml^{-1} propidium iodide at 25°C for 30 minutes and analysed by FACScan flow cytometer with Cell Quest software (Becton Dickinson, Braintree, MA).

Microtubule co-sedimentation assay

The microtubule co-sedimentation assay was performed as described previously with slight modification (Piddini et al., 2001). Briefly, PC12 cells that had been cultured in 100-mm dishes were lysed in 0.2 ml of an ice-cold solubilizing buffer [80 mM K-PIPES (pH 6.8), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT, 1 mM GTP, 1% (w/v) NP-40, 2 μg ml^{-1} aprotinin, 0.5 mM Pefabloc SC] and clarified by centrifugation at 200,000 ***g*** for 30 minutes at 4°C. The supernatant was split into two samples, one of which was kept on ice and the other supplemented with 40 mM Taxol (Sigma) and incubated for 30 minutes at 37°C. Both samples were spun through a 10% sucrose cushion at 100,000 ***g*** for 10 minutes at 25°C. The resulting pellets and supernatants were resuspended in SDS-PAGE loading buffer.

All experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

DDBL/EMBL/GenBank accession numbers

The accession numbers for Gie1 and Gie2 are AB118751 and AB118752, respectively.

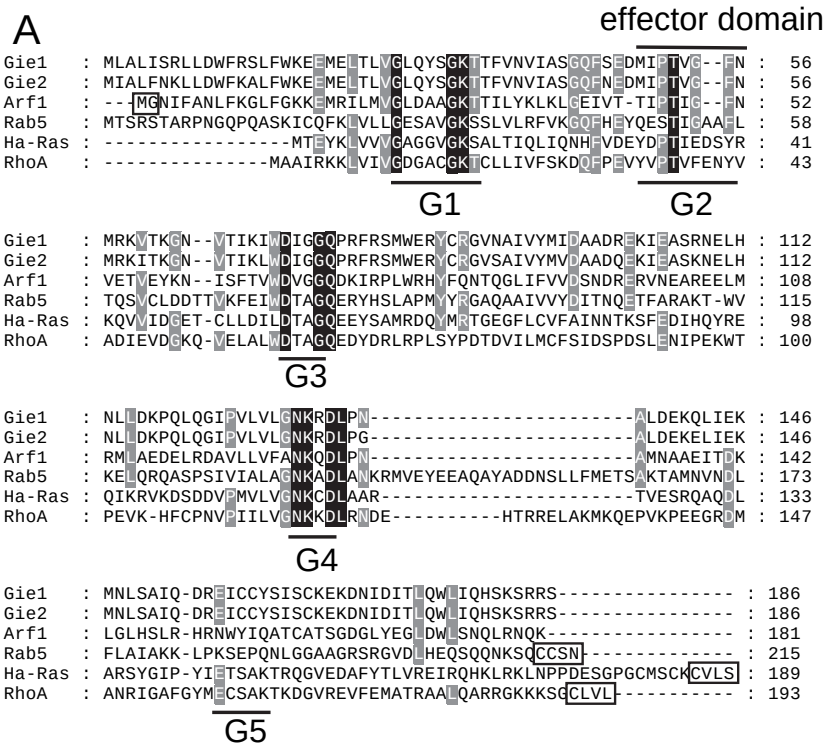
Results

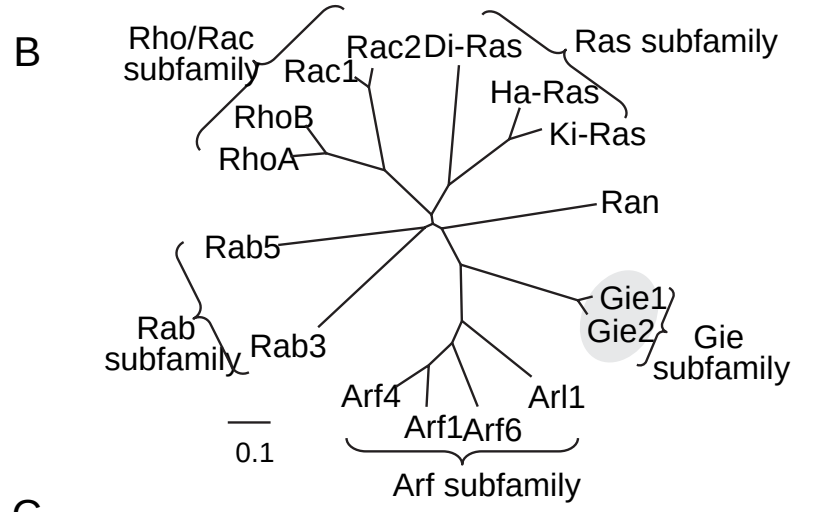
Identification of Gie as a novel subfamily of the small GTPases

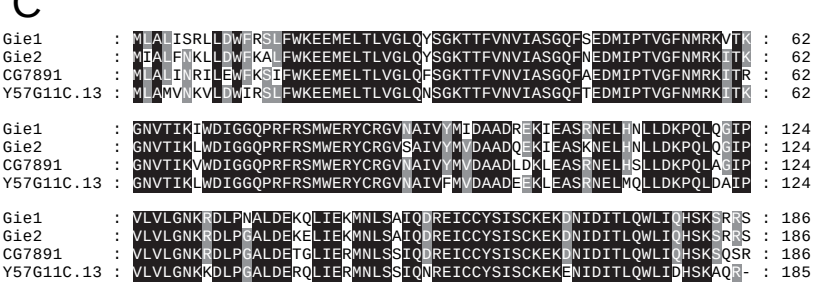
Gie1 was first identified in searching human genome database for new GTPases as follows. We found that a cosmid clone derived from the chromosome 3p26.1 locus contains an open reading frame that encodes a putative Ras-related protein, which we designated hGie1. We also found a sequence encoding another GTPase (a cosmid clone derived from the chromosome 1q32.1 locus) that is closely related to hGie1 (91% identity, 98% similarity in amino acid sequence). Thus, we designated the second protein hGie2. To confirm the actual transcription of these genes, PCR analysis was performed using human brain cDNA library. We obtained PCR products with the expected molecular weights, of which nucleotide sequences matched the coding regions within the cosmid DNAs. There are some unpublished DNA sequences in the EMBL database, of which coding sequences are identical to *hGie1* and *hGie2*.

Amino acid alignment shows that hGie1 and hGie2 share only about 30% amino acid identity with other members of the small GTPases (Fig. 1A). Motif searches of the predicted Gie sequences revealed that they contain highly conserved GTP-binding domains (G1-G5) and a putative effecter domain (corresponding to the amino acid sequence 32-40 of Ha-Ras) (Marshall, 1996). However, there are no obvious

lipid-modification motifs in human Gie proteins: Neither a CAAX motif (where C is Cys, A is an aliphatic amino acid and X is any amino acid) nor an MG motif (M is first Met and G is Gly) is present at their C- or N-termini (Magee et al., 1992; Moss and Vaughan, 1995). An unrooted phylogenetic tree of Gie and other small GTPase members indicates that Gie1 and Gie2 can be classified into a distinct GTPase subfamily (Fig. 1B). We found several overlapping clones of mouse and rat ESTs whose primary structures are highly homologous to that of hGie1 (data not shown). In addition, BLAST search of the protein database (Altschul et al., 1997) revealed that there are Gie homologs in *Drosophila melanogaster* (CG7891) and *C. elegans* (Y57G11C.13) (Fig. 1C). However, we could not find out any Gie homolog in yeast, indicating that Gie is well conserved in multicellular organisms. No functional correspondence of these genes has yet been established.







Gie is ubiquitously expressed in various tissues and cell lines

In order to determine the expression pattern of hGie mRNAs, northern blot analysis was performed in human tissues. We found that two transcripts (3.5 kb and 2.0 kb) of *Gie1* were expressed in the various tissues and that higher levels of the expression were observed in the brain, heart, skeletal muscle, kidney and placenta (Fig. 2A, top). *Gie2* mRNA (2.0 kb) was also expressed ubiquitously (Fig. 2A, middle). Although the smaller size of the *Gie1* transcripts appeared to be similar to the 2.0-kb *Gie2*, it is unlikely that *hGie1* probe detected the *Gie2* mRNA because the expression patterns of the 2.0-kb *Gie1* and *Gie2* were different. We also raised an antibody that can recognize both Gie1 and Gie2. This anti-Gie antibody was capable of recognizing a 22-kDa polypeptide in rat PC12 cells, and absorption of the antibody with the

Fig. 1. Identification of Gie as a novel subfamily of the small GTPases. (A) Comparison of amino-acid sequences of human Gie1, Gie2 and other small GTPase members. The amino-acid sequences were aligned using the ClustalW program. Amino-acid residues conserved among all the proteins are shaded in black, and those with 66-99% identity are in light gray. G1-G5 indicate well-conserved regions important for binding to phosphate/Mg^{2+} and the guanine base. The region considered to be a putative effecter domain is overlined. The C-terminal sequence for prenylation (CAAX box: A, aliphatic; X, any amino acid) and the N-terminal sequence for myristoylation (MG box) are boxed. The human sequence data are available from GenBank/EMBL/DDBJ under the accession numbers AK001564 (Gie1) and BC015408 (Gie2). (B) An unrooted tree constructed using ClustalW with standard parameters and viewed using TreeView. (C) Deduced amino-acid sequences from the indicated genes were aligned using the ClustalW. Amino-acid residues conserved among all the proteins are shaded in black, and those with 75-99% identity are in light gray. The accession numbers of *Drosophila melanogaster* CG7891 and *Caenorhabditis elegans* Y57G11C.13 are NP649769 and CAB16514, respectively, in the NCBI Entrez Protein database.

antigen peptide abolished the reactivity (Fig. 2B). This antibody was useful for identifying endogenous Gie proteins in various tissues and cell lines (Fig. 2C); the expression level of Gie proteins was rather low in HeLa cells compared with other cell lines, including PC12 cells.

Biochemical properties of Gie proteins

We next investigated whether Gie proteins were capable of binding to GTP/GDP in living cells. For the analysis, FLAG-tagged hGie1 and hGie2 were expressed in HeLa cells, and the proteins were purified by means of immunoprecipitation. Expression of the transfected constructs was confirmed by immunoblotting and the guanine nucleotides associating with the immune complex were analysed by thin-layer chromatography. As reported previously, wild-type Ha-Ras and the G12V mutant existed predominantly as GDP-bound and GTP-bound forms, respectively (Fig. 3A, left). By contrast, both the nucleotide-bound forms were clearly observed in wild-type hGie1 and hGie2 (Fig. 3A, middle). These results indicate that Gie proteins also cycle between GTP-bound and GDP-bound forms in living cells. In addition, we could obtain three unique mutants of Gie1 (W70R, T34N and N130I) that correspond to the Arf1 mutants W66R, T31N and N126I (Kahn et al., 1995; Peters et al., 1995). Gie1/W70R and Gie1/T34N preferred GTP and GDP for their binding, respectively, whereas Gie1/N130I was characterized as a nucleotide-free form (Fig. 3A, right). Based on the findings of Arf1 mutants, it is very likely that the W70R and T34N mutants have defects in GTPase and GTP-GDP exchange reactions, respectively, whereas the N130I mutant lacks GTP/GDP-binding activity. Thus, the T34N and N130I mutants are expected to exhibit dominant-negative phenotypes by sequestering GEFs from the endogenous proteins, when expressed in cells.

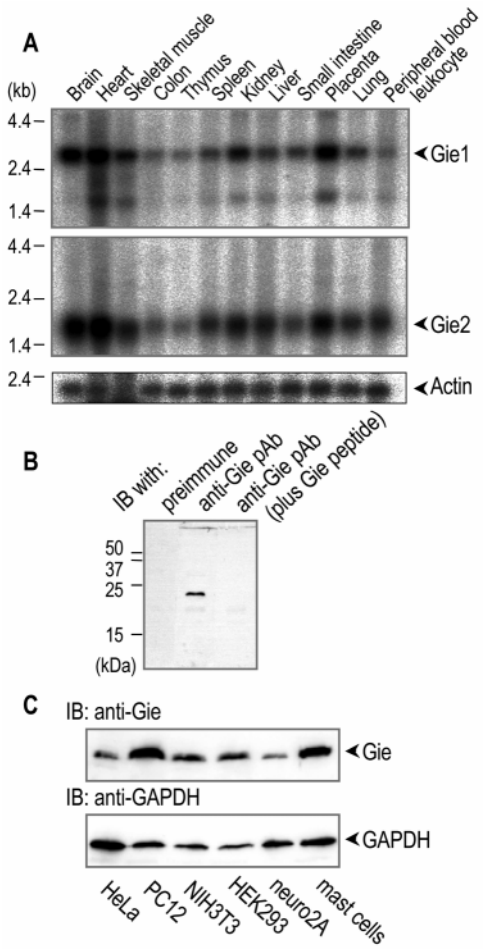


Fig. 2. Expression of Gie in various human tissues and cell lines. (A) Expression of *hGie1* and *hGie2* mRNAs in human tissues. Northern blots of poly(A)$^+$ RNAs from various human tissues (Clontech) were hybridized with ^{32}P-labeled Gie probes. (B) Extracts from PC12 cells were separated by SDS-PAGE and immunoblotted with pre-immune serum (left), the anti-Gie antibody (middle) or the same antibody that had been incubated with the antigen peptide (right). (C) Extracts from the indicated cell lines were also immunoblotted with the anti-Gie (top) and anti-GAPDH (bottom) antibodies.

Overexpression of dominant-negative Gie mutants induces abnormal chromosomes

To determine the roles of Gie, HeLa cells were transfected with expression vectors carrying the various *Gie1* mutants (Fig. 3B). There was no apparent effect on the cell morphology upon the expression of wild-type Gie1 (Fig. 3B, second panels) or the W70R mutant (Fig. 3B, third panels). The expressed Gie proteins were widely distributed in the cytoplasm. However, overexpression of either Gie1/T34N or Gie1/N130I caused abnormal chromosomes. The appearance of micronuclei, which might be analogous to the phenotype of fission yeast *cut* mutants (Yanagida, 1998), were abundantly observed in the interphase of HeLa cells (Fig. 3B, fourth and fifth panels, arrows). The formation of these tiny nuclear structures was likely to be the consequence of chromosome mis-segregation but not of a cytokinesis defect, because the fluorescence-activated cell sorting analysis of the cells expressing dominant negative forms of Gie1 showed no increase in polyploidy (data not shown). The remainder of the cells, however, divided normally, presumably reflecting difference in expression levels. The degree of the aberrant nuclei was statistically analysed under the various conditions (Fig. 3C). The ratio of nuclear abnormality was significantly higher in Gie1/T34N- or Gie1/N130I-expressing cells than in Gie1/wild-type- or Gie1/W70R-treated cells. Thus, human Gie appears to play a role in chromosome segregation.

Complete sister-chromosome segregation is inhibited by RNAi of *Drosophila* Gie

To investigate whether the role of Gie observed in the mammalian cells is evolutionarily conserved, RNAi was used in *Drosophila* S2 cells. S2 cells at an exponentially growing stage were cultured with dsRNA for 4 days, and the amount of *Drosophila* Gie (*dGie*) mRNA was analysed by semi-quantitative PCR. There was marked reduction of *dGie* mRNA in the dsRNA-treated cells compared with the control cells, although the Aurora-B signals used as loading markers were the same (Fig. 4A). To assess the nature of defects in cell-cycle progression, we stained cells to reveal DNA and microtubules at 4 days after the treatment with *dGie* dsRNA. The flow-cytometry analysis showed that the mitotic index of

dGie-knockdown cells was comparable to that of control cells (Fig. 4B), indicating that mitotic progression was not delayed by the *dGie* RNAi. However, immunofluorescence microscopy of S2 cells revealed that, although cells reach metaphase normally, approximately 40% of anaphase cells that contain the spindle and separated chromosomes displayed aberrant morphology, as indicated by the increased number of anaphase cells holding chromatin bridges (Fig. 4C, second panels, Fig. 4D, lower panels). It was also noticeable that a small proportion of the cells occasionally contained lagging chromosomes (Fig. 4C, third panels and Fig. 4D, lower panels). These results indicated that the reduction of *dGie* levels led to chromosome mis-segregation but did not inhibit mitotic progression. These chromosome abnormalities are comparable to the phenotype observed with the dominant-negative mutants (Gie1/T34N and Gie1/N130I) in HeLa cells. Thus, the function of Gie in chromosome segregation appears to be evolutionarily conserved in higher eukaryotes.

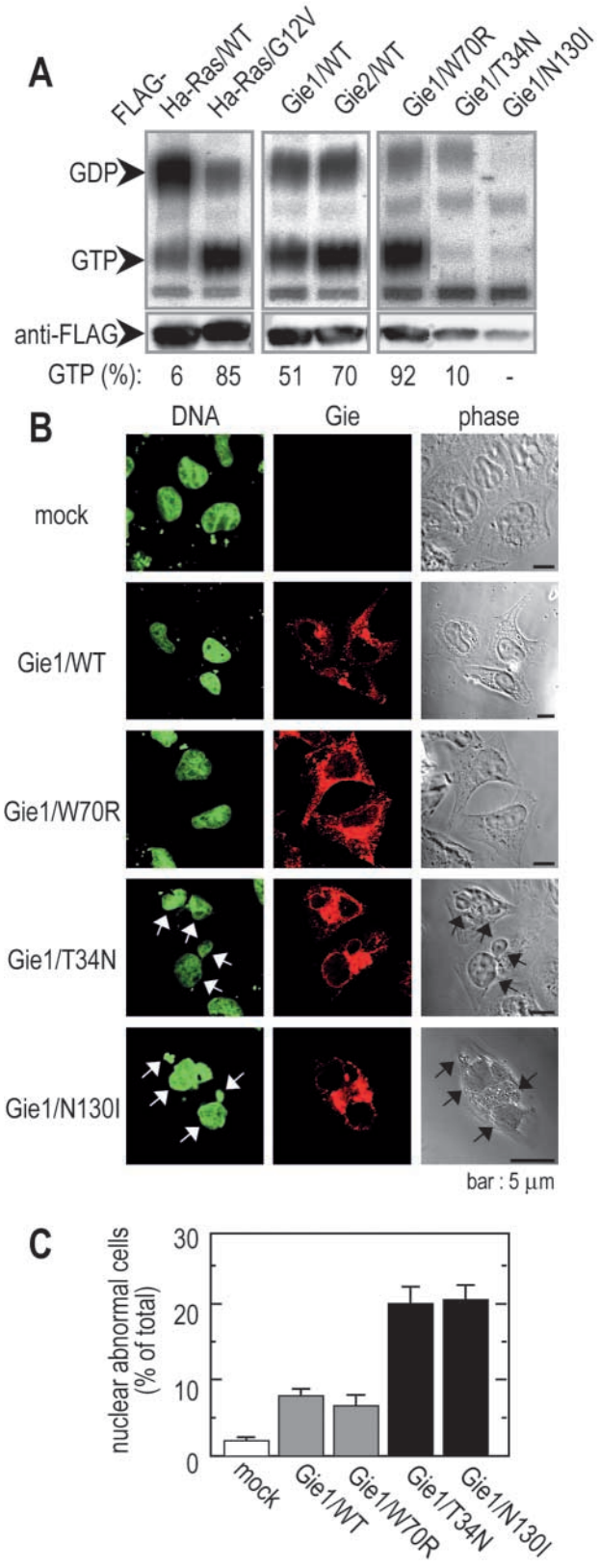


Fig. 3. Overexpression of dominant-negative Gie mutants induces abnormal morphology in the chromosomes of HeLa cells. (A) Identification of nucleotide-bound forms of Gie1 and Gie2. HeLa cells were transfected with expression vectors encoding the FLAG-proteins listed at the top and metabolically radiolabeled with ^{32}P. The expressed proteins were immunoprecipitated with an anti-FLAG monoclonal antibody, and nucleotides associating with the proteins were separated by thin-layer chromatography. The radioactivity of GTP and GDP was quantified, and the proportions of GTP-bound form in total Gie proteins are shown at the bottom. (B) HeLa cells were grown on poly-L-lysine-coated coverglasses and transfected with the various mutants of Gie1 or with pCMV5 vector alone. DNA (green) and Gie1 (red) were detected with PicoGreen and the anti-Gie antibody, respectively. Phase-contrast photographs images (right) are also shown. Most of the cells expressing Gie1/T34N and Gie1/N130I exhibited phenotypes characterized as micronuclei (white arrows). Scale bar, 5 μm. (C) The appearance of abnormal nuclei was measured, and the data are represented as percentages of means±s.e.m. from at least three independent experiments (each of 200-400 cells).

Localization of Gie during the cell cycle

To examine the localization of endogenous Gie in different stages of cell cycle, PC12 cells (which contain a high level of Gie) were subjected to immunofluorescence analysis with the affinity-purified anti-Gie antibody. DNA and microtubules were also stained with DAPI and an anti-α-tubulin antibody, respectively. Based on the cell morphology and tubulin staining, we could pick up various cell-cycle stages of PC12 cells including interphase, prometaphase, metaphase, anaphase and telophase/cytokinesis. Double immunofluorescence staining showed that, during interphase, Gie localized mainly to the perinuclear region, where tubulin was focused on microtubule-organizing centers (Fig. 5, first panels). In prometaphase and metaphase, Gie spread throughout the cytoplasm but was excluded from the mitotic spindles and the chromosomes (Fig. 5, second and third panels). During early anaphase, Gie was localized predominantly to part of the cell cortex, and a subpopulation of Gie accumulated in the spindle mid-zone (Fig. 5, fourth panel). In late anaphase and telophase, Gie formed a distinct fine band extending across the spindle mid-zone and became more sharply concentrated on the mid-body except its center as the cells progressed to cytokinesis (Fig. 5, fifth and sixth panels).

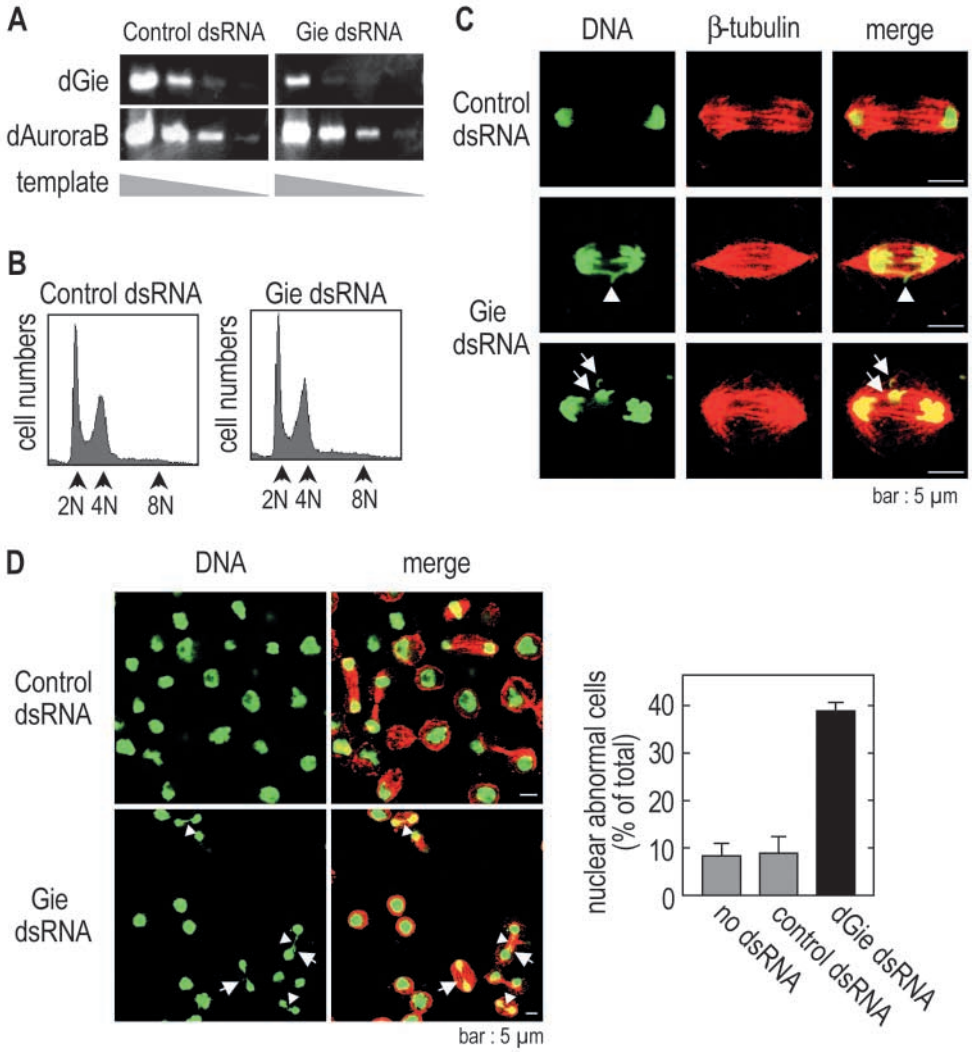


Fig. 4. *Drosophila* Gie is required for chromosome segregation. (A) Reduction of *dGie* mRNA levels by RNAi. *Drosophila* S2 cells were cultured with the control dsRNA (left) or *dGie* dsRNA (right) for 4 days, and the level of *dGie* transcript was measured by semiquantitative reverse-transcription PCR with decreasing amounts of the templates. *Aurora-B* mRNA was used as a loading control. (B) Flow cytometry analyses of the control (left) and *dGie*-dsRNA-treated (right) S2 cells. (C) The control (top) and dsRNA-treated (middle and bottom) S2 cells were stained with PicoGreen and the anti-α-tubulin antibody to reveal DNA (green) and microtubules (red), respectively. Gie-depleted S2 cells displayed no separation of their chromatids before cytokinesis and remained with a thin chromatin string (arrowhead). They also had lagging chromosomes (arrows). Scale bar, 5 μm. (D, left) Representative immuno-stains of control (top) and *dGie*-dsRNA-treated S2 cells (bottom). The appearance of cells that had a chromatin string (arrowhead) or lagging chromosomes (arrows) at anaphase stage was measured. (D, right) The data are represented as percentages of means±s.e.m. from at least three independent experiments (each of 100 cells).

Gie associates with microtubules independent of its guanine-nucleotide-binding forms

The co-localization of Gie with microtubules during anaphase and telophase suggests a specific interaction between Gie and tubulin. To determine whether Gie associates physically with microtubules, HeLa cells expressing FLAG-tagged wild-type Gie1 and Ha-Ras were immunoprecipitated with the anti-FLAG antibody and subjected to protein staining and immunoblotting. FLAG-tagged Gie1, but not FLAG-tagged Ha-Ras, appeared to associate with a 55-kDa protein (Fig. 6A, left), and the protein reacted with an anti-β-tubulin antibody (Fig. 6A, right). Other than the 55-kDa protein, there were no major bands reactive to FLAG-tagged Gie1, excluding the possibility that the interaction between Gie and tubulin was mediated through other proteins. Further analysis indicated that Gie1 is capable of interacting with α- and β-tubulin, but not with γ-tubulin (data not shown). We further characterized the Gie-tubulin interaction in co-sedimentation experiments. Gie proteins were co-sedimented specifically with Taxol-stabilized microtubules. By contrast, the presence of Gie in the pellet fraction was negligible when microtubule polymerization was inhibited by keeping the tube on ice (Fig. 6B). In control experiments, Rab5 did not associate with Taxol-polymerized microtubules (Nielsen et al., 1999). This finding that Gie proteins bind to polymerized microtubules is consistent with the Gie localization during anaphase and telophase (Fig. 5).

We next investigated whether the tubulin-binding ability was dependent on the nucleotide-bound forms of Gie. For the analysis, FLAG-tagged wild-type Gie1 and the various mutants were expressed in HeLa cells, and their tubulin-binding abilities were investigated using the immunoprecipitation assay. All the Gie1 mutants interacted with β-tubulin (Fig. 6C), indicating that the association is independent of the nucleotide-bound forms. Thus, it is unlikely that tubulin is a downstream effector for Gie. Generally, GTPases contain at least two switch regions whose conformation is regulated by the bound guanine nucleotide, and roles of the switch regions have been extensively studied in Ras and Arf proteins (Goldberg, 1999; Kuai et al., 2000). Alterations in these two regions result in changes in the affinity of small GTPases for their effectors or regulatory proteins, such as GEFs and GAPs. To verify that tubulin is a binding partner of Gie rather than its effector or regulator, we further generated two additional Gie1 mutants that lack the putative switch I (residues 49-58) or switch II (residues 74-

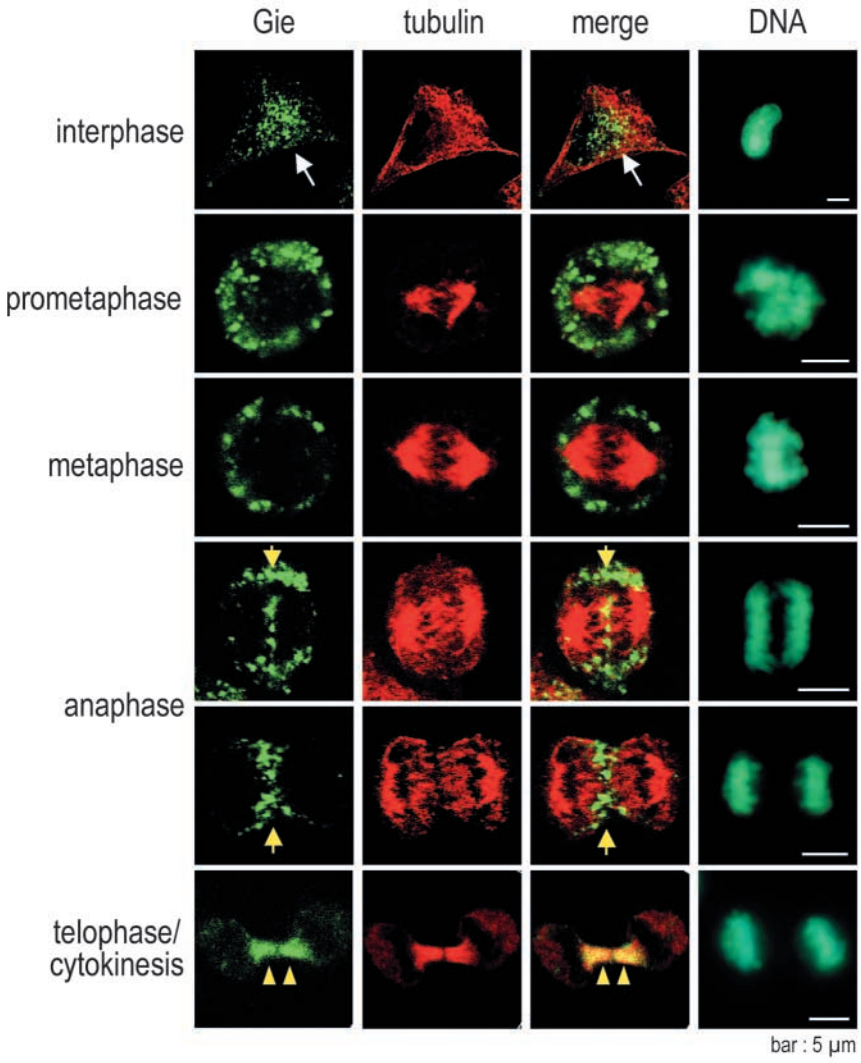


Fig. 5. Cell-cycle-dependent dynamics of Gie localization. PC12 cells were fixed and stained for endogenous Gie with the affinity-purified anti-Gie antibody (green). Microtubules (red) and DNA (blue) were also visualized with anti-α-tubulin antibody and DAPI, respectively. Gie displayed cytoplasmic distribution in interphase (white arrows) but moved prominently to the spindle mid-zone (yellow arrows) in anaphase and to the mid-body in late-telophase (yellow arrowhead). Scale bar, 5 μm.

85) region and examined their tubulin-binding abilities using the immunoprecipitation assay. As expected, both mutants could interact with tubulin (Fig. 6D).

Gie mutants lacking switch regions are dominant negative in chromosome segregation

Although the above two mutants are still capable of interacting with tubulin, they might function in a dominant-negative manner because of the lack of an effector domain. To test this prediction, we examined the chromosome segregation in HeLa cells expressing these Gie mutants. Overexpression of the mutants lacking the switch regions caused extensive abnormality in the chromosome morphology, which is characterized by micronucleus formation (Fig. 7A). The abnormality was significantly more common in HeLa cells expressing the Gie mutants than in vector-treated or wild-type Gie1-expressed cells (Fig. 7B). These results suggest that correct functions of Gie in chromosome segregation require its putative switch (or effector) regions as well as its interaction with microtubules.

Discussion

In the present study, we have identified human Gie1 and Gie2, which form a new distinct subfamily of the small GTPases. This subfamily is well conserved in multicellular organisms. Expression of dominant-negative Gie mutants in human HeLa cells or knockdown of Gie transcripts by RNAi in *Drosophila* S2 cells induced abnormal morphology in the chromosome segregation. Gie was capable of associating with microtubules in living cells, and this association appeared to be direct and independent of GTP- and GDP-bound forms of the small GTPase. Furthermore, overexpression of Gie mutants lacking its putative effector domains also impaired the chromosome morphology. The present data suggest that Gie might play an indispensable role in the equal segregation of chromosomes, probably through its association with microtubules, although it remains unknown how Gie regulates chromosome segregation.

Gie forms a new small GTPase subfamily

Based on database and phylogenetic analyses, Gie appears to be part of a new conserved subfamily of the small GTPases that so far contains two human, one *Drosophila* and one *C.*

elegans members. However, Gie does not exist in yeast, indicating that its function might be required for multicellular organisms. Gie subfamily differs from most other small GTPase families in the lack of lipid-modification motifs, indicating that Gie functions without associating with lipid membranes. Most small GTPases have sequences at their N- or C-termini that undergo post-translational modifications with lipids such as myristic acid or farnesyl, geranylgeranyl and palmitoyl methyl moieties. Another family, Ran, also does not have such sequences to direct post-translational modifications. Thus, Ran proteins localize to either the cytosol or the nucleus, although most small GTPases exist in the membranes or complexes with GDP-dissociation inhibitors (GDIs) in the cytosol. In mammalian cells, Gie appears to localize to the cytosol but not to a specific membrane (Fig. 5), consisting with the idea that this subfamily might not have the post-translational modification with lipid.

Gie is required for chromosome segregation

We found that the expression of dominant-negative Gie mutants in human HeLa cells induced abnormal chromosomes (Fig. 3), indicating that inhibition of Gie function in living cells causes serious defects in chromosome function. We first performed RNAi experiments in HeLa and PC12 cells but failed to inhibit the expression of Gie because of the existence of two homologs in the mammalian cells. Instead, we depleted

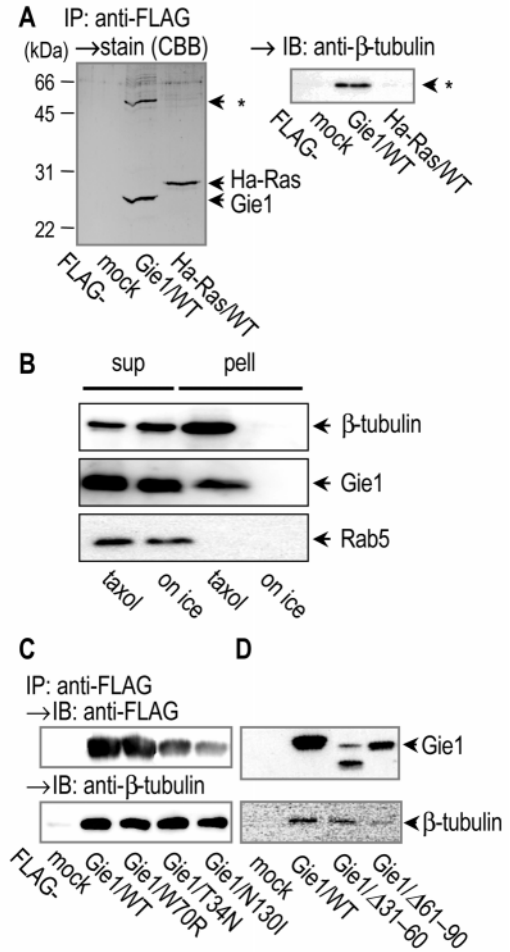


Fig. 6. Gie associates with tubulin independent of its guanine-nucleotide-bound forms or the absence of its effector domains. (A) Extracts from HeLa cells expressing FLAG-tagged wild-type Gie1 and Ha-Ras were subjected to immunoprecipitation (IP) with the anti-FLAG antibody. The precipitants were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (left) or immunoblotting (IB) with an anti-β-tubulin antibody (right). The asterisk denotes the position of interacting protein. (B) Microtubule co-sedimentation assay. Gie stayed mainly soluble (sup) when microtubule polymerization was inhibited by keeping on ice (lanes 2,4), and was specifically recovered in the pellet (pell) in the presence of Taxol-stabilized microtubules (lanes 1,3). The behavior of the Rab5 is shown as a negative control. (C,D) HeLa cells were transfected with expression vectors encoding the proteins listed at the bottom and the cell extracts were immunoprecipitated with the anti-FLAG antibody. The precipitants were subjected to immunoblotting using anti-FLAG and anti-β-tubulin antibodies.

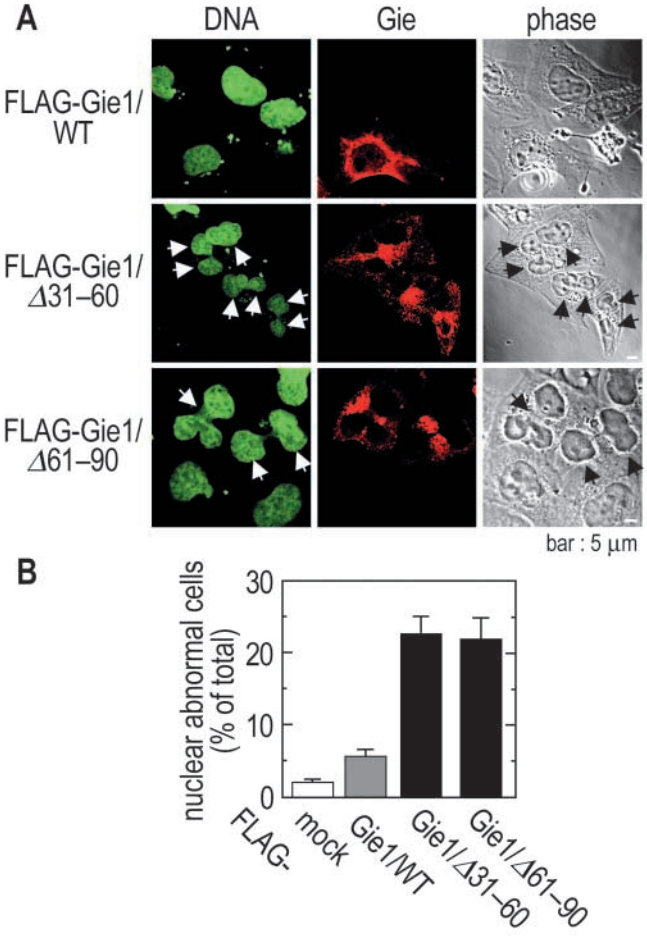


Fig. 7. Overexpression of Gie1 mutants lacking the switch regions impairs chromosome segregation in HeLa cells. (A) HeLa cells that had been transfected with the indicated Gie1 and cultured for 24-30 hours were analysed by immunofluorescence microscopy. DNA (green) and Gie1 (red) were detected with PicoGreen and the anti-Gie antibody, respectively. Impaired chromosome segregation was observed in cells expressing Gie1/Δ31–60 and Gie1/Δ61–90 (white arrows). Scale bar, 5 μm. (B) The appearance of abnormal nuclei was measured and the data are represented as percentages of means±s.e.m. from at least three independent experiments (each of 200-400 cells).

endogenous Gie by adapting RNAi methods in *Drosophila* S2 cells. Although S2 cells treated with a *dGie* dsRNA underwent mitosis and cytokinesis, they frequently failed to separate all their chromatids and remained connected by a thin thread of chromatin (Fig. 4C,D). The chromatin bridge and lagging chromosomes at anaphase reflect the structural instability of the chromosomes. This phenotype is reminiscent of the *cut* phenotype of fission yeast. It should be realized that the mammalian cells transfected with non-degradable securin divide but daughter nuclei remain connected by chromatin (Zur and Brandeis, 2001), similar to the phenotype caused by *dGie* RNAi. Another similar phenotype has been also observed in nematode embryos that are depleted of the nuclear lamina protein lamin (Liu et al., 2000), in *Drosophila* S2 cells lacking either Aurora B or topoisomerase II (Chang et al., 2003; Giet and Glover, 2001), and in mammalian cells lacking condensin (Hudson et al., 2003). Thus, it is very interesting to know the relationship between Gie and these factors involved in chromosome segregation.

Gie associates with α- and β-tubulins

Immunohistochemical studies revealed that, during interphase, Gie localized to the cytoplasm along with microtubules, then redistributed to the spindle mid-zone in anaphase and to the mid-body in late telophase (Fig. 5). This distribution implied that Gie might interact with microtubules. In fact, we found that Gie interacts with α- and β-tubulin in co-immunoprecipitation and co-sedimentation experiments (Fig. 6). The association of Gie with α- and β-tubulins prompted us to examine whether Gie also binds to γ-tubulin, but we could not detect any association between Gie and γ-tubulin.

Co-localization between Gie and microtubules suggests that Gie might play another role at different stages of cell cycle through its association with microtubules. The head-to-tail arrangement of α- and β-tubulin within the microtubule confers structural polarity on the polymer. The radial array of microtubules in an interphase cell is largely organized by the centrosome, with the microtubule minus ends at the centrosome and the plus ends extending out towards the cell periphery. Microtubules exist in dynamic equilibrium with tubulin subunits, growing and shrinking by the addition or deletion of tubulin dimers from the ends of the microtubules (Kirschner and Mitchison, 1986). Individual microtubules switch between phases of slow growth and rapid shrinkage so that, in a microtubule population, some will be growing and some shrinking, a property known as dynamic instability (Hyman and Karsenti, 1996; Mitchison and Kirschner, 1984; Walker et al., 1988). However, there have been no reports showing that small GTPases bind directly to tubulin. Therefore, Gie might serve as a switch between the polymerization and depolymerization of microtubules. In relation to this, we have preliminarily observed that overexpression of the constitutively active mutant of Gie1/W70R induces cell extension in HeLa cells, and this phenotype is inhibited by the cell treatment with nocodazole but not with cytochalasin B (data not shown). Thus, it is tempting to speculate that Gie might have different roles depending on the cell types or cell-cycle conditions. This prediction is consistent with the data that Gie is highly expressed in non-dividing tissues such as brain or heart (Fig. 2).

Gie localizes to the mid-body in late mitosis

During anaphase and telophase, Gie localizes to the spindle mid-zone and concentrates at the mid-body. Several proteins accumulate at the central spindle of mammalian mitotic cells and have been shown to play roles in cytokinesis and mitotic events including spindle formation or alignment and chromosome segregation (Glotzer, 2001; Guertin et al., 2002; Scholey et al., 2003). Some interactions among these proteins have been already established, but their specifically defined functions in the cell-division process are still largely unknown. Gie might serve as a molecular switch to regulate chromosome segregation through the association with microtubules in a certain signaling pathway.

We also found that the association of Gie with tubulin is independent of its nucleotide-binding forms. This indicates that tubulin is not categorized as an effector for Gie (like Raf for Ras proteins). Because tubulin has been reported to mediate the translocation of various proteins, it is very likely that Gie is allowed to use the microtubule network for its translocation in the mitotic phase, owing to its tubulin-binding ability. However, the interaction between Gie and microtubules appeared to be dependent on the stage of cell cycle. The exclusion of Gie from mitotic spindles during early mitosis suggests that its ability to associate with microtubules is specifically inhibited. By contrast, Gie strongly colocalizes with microtubules during late mitosis and interphase. These observations indicate that the microtubule-binding ability of Gie might be masked during early mitosis and unmasked during the late stages, probably through some modification when it localizes to the spindle mid-zone and mid-body. In relation to this, we have preliminary data suggesting that Gie is phosphorylated upon the expression in HeLa cells (data not shown). Recently, various protein kinases localizing to the mid-body have been isolated from vertebrate cells (Nigg, 2001). Importantly, however, targets of these kinases remain largely unknown. An attractive and possible explanation for our observations is that phosphorylation of Gie by a certain kinase(s) specifically regulates its microtubule-binding ability in mitosis.

In summary, this is the first report showing that a small GTPase subfamily, here termed Gie, might be involved in chromosome segregation. Recently, there have been several notable developments in the field of chromosome segregation. However, a molecular mechanism underlying the signaling pathway still remains elusive. Thus, further studies of Gie, including the identification of its effector(s) (which might regulate cell division and localize to the spindle mid-zone and mid-body) would provide new insights into the molecular basis of chromosome segregation.

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