MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody.

Koichi Hirose\textsuperscript{1,2}, Toshiyuki Kawashima\textsuperscript{1}, Itsuo Iwamoto\textsuperscript{2}, Tetsuya Nosaka\textsuperscript{1}, and Toshio Kitamura\textsuperscript{1,3}

\textsuperscript{1} Division of Hematopoietic Factors, The Institute of Medical Science, University of Tokyo, minato-ku Tokyo 108-8639,

\textsuperscript{2} Department of Internal Medicine II, Chiba University School of Medicine, Chiba 260-0856, Japan

\textsuperscript{3} Corresponding author
TEL: 81-3-5449-5758
FAX: 81-3-5449-5453
E-mail: kitamura@ims.u-tokyo.ac.jp
Summery

We have recently cloned a cDNA for a full-length form of MgcRacGAP. Here we show using anti-MgcRacGAP antibodies that, unlike other known GAPs for Rho family, MgcRacGAP localized to the nucleus in interphase, accumulated to the mitotic spindle in metaphase, and was condensed in the midbody during cytokinesis. Overexpression of an N-terminal deletion mutant resulted in the production of multinucleated cells in HeLa cells. This mutant lost the ability to localize in the mitotic spindle and midbody. MgcRacGAP was also found to bind α-, β-, γ-tubulins through its N-terminal myosin-like domain. These results indicate that MgcRacGAP dynamically moves during cell cycle progression probably through binding to tubulins and plays critical roles in cytokinesis. Furthermore, using a GAP-inactive mutant, we have shown that the GAP activity of MgcRacGAP is required for cytokinesis, suggesting that inactivation of Rho family of GTPases may be required for normal progression of cytokinesis.
Introduction

A cell divides into two daughter cells through nuclear division and cytokinesis. In cytokinesis, formation of an actomyosin-based contraction ring separates the cytoplasm to two daughter cells (1,2). A small GTPase RhoA localizes in the cleavage furrow during cytokinesis and plays important roles in cytokinesis (3). In Xenopus eggs, microinjection of a Rho specific inhibitor C3, an exoenzyme from Clostridium botulinum, prevents the progression of cytokinesis (4). Other members of the Rho family of GTPases may also be involved in cytokinesis. A human cell line expressing a constitutively active mutant of Cdc42, as well as a Dictyostelium strain lacking the RacE gene encoding a Rac/Cdc42 related protein, produced multinucleated cells as a result of impaired cytokinesis (5,6). Recently, putative cytoplasmic targets of the Rho family of GTPases have been identified, and some of which, including Gic, Bni 1p, and Citron kinase, seem to be specifically involved in cytokinesis (7-10).

The Rho family of small GTPases, represented by RhoA, Rac, and Cdc42, act as molecular switches of diverse biological functions involving remodeling of cytoplasmic actin and microtubules (11,12). The GTP-bound Rho protein is an active form, while the GDP-bound form is inactive. Activation of the Rho family is promoted by guanine nucleotide exchange factors (GEFs), which catalyze the replacement of bound GDP by GTP. The GTP-bound form of the Rho family can specifically interact with their effectors or targets and transmit signals to downstream molecules. On the other hand, they are inactivated through hydrolysis of bound GTP to GDP by their intrinsic GTPase activities, assisted by GTPase activating proteins (GAPs) (13). Several GEFs are known to play roles in cytokinesis. For example Vav-3, a novel Vav family protein which has a GEF activity for RhoA, RhoG and Rac, has an important role in cytokinesis (14). Another GEF for the Rho family, ECT2, is implicated in cytokinesis (15). In Drosophila, Pbl, a homologue of mammalian ECT2, was found to be required for initiation of cytokinesis (16). On the other hand, which GAPs inactivate the Rho family of GTPases involved in cytokinesis has been largely unknown.

3
In a search for molecules involved in macrophage differentiation, we identified a cDNA for Rac/Cdc42-specific GAP, whose anti-sense cDNA inhibited macrophage differentiation of mouse leukemic M1 cells induced by IL-6. Moreover its overexpression induced differentiation of human leukemic HL-60 cells into macrophage. This Rac/Cdc42-specific GAP is a homologue of a previously identified RacGAP, MgcRacGAP (17). However, our clone encoded an additional 106 amino acids at the N-terminus that has a myosin-like sequence (18). In fact, the human counterpart we identified had a similar myosin-like domain, indicating that the previously reported cDNA for human MgcRacGAP encoded a protein with an N-terminal deletion. Overexpression of MgcRacGAP retarded proliferation and induced formation of multinucleated cells in all cell lines examined. In addition, the highest expression level of MgcRacGAP mRNA was observed at the G2/M phase. These data prompted us to determine if MgcRacGAP regulates cell proliferation through the control of cytokinesis. Immunohistochemical studies showed that MgcRacGAP co-localized with the mitotic spindle in metaphase, and was transferred to the midzone in anaphase and telophase, and moved to the midbody in cytokinesis. Analysis of deletion mutants of MgcRacGAP showed that overexpression of a myosin-like domain-deletion mutant or a GAP activity-defective mutant of MgcRacGAP halted cell division and led to form multinucleated cells. We also showed that MgcRacGAP associated with microtubules in vivo through the N-terminal myosin-like domain. These data indicated that MgcRacGAP plays key roles in the cell cycle machinery, especially in the G2/M phase, through binding to microtubules.
Experimental procedures

Immunostaining

HeLa cells were plated on glass coverslips, and the next day the cells were washed three times with ice cold PBS and fixed with 4% paraformaldehyde/PBS for 20 min at room temperature. The cells were washed three times with ice-cold PBS followed by a 10 min incubation at room temperature in PBS containing 0.1% Nonidet P-40. The permeabilized cells were washed three times with ice cold PBS. The antibodies, anti mouse α-tubulin (SIGMA clone No. DM 1A), mouse anti-Flag (M2), and rabbit anti-Flag (Zymed) were diluted in PBS containing 3% bovine serum albumin (BSA), placed as a drop on the coverslips, and incubated for 1 hour at 37°C. The coverslips were washed six times with PBS and covered with a solution containing FITC-conjugated goat anti-rabbit Ig (Wako), Rhodamine-conjugated goat anti-mouse Ig (SIGMA), and DAPI (4’ 6-diamino-2-phenylindole) at 1μg/ml for 30 min. in the dark at 37°C. The coverslips were then washed six times with PBS. After the final wash, the coverslips were mounted with glycerin containing para-phenylenediamine (PPD) at 10mg/ml and viewed with a fluorescence microscope IX70 (Olympus) equipped with SenSys/OL cold CCD camera (Olympus) and IP-Lab software (Signal Analytics Co.).

Cell culture and transfection

HeLa cells were grown in DMEM supplemented with 5% fetal calf serum (FCS) and seeded into a 6 cm dish at 1×10^6/dish. On the following day, HeLa cells were transiently transfected with plasmids encoding the wild type or the mutant forms of MgcRacGAP using LipofectAMINE-PLUS (Gibco-BRL) according to manufacturer’s recommendations. BaF/3 cells were grown in RPMI 1640 supplemented with 10% FCS and 2 ng/ml of mouse IL-3. HL-60 and Jurkat cells were grown in RPMI 1640 supplemented with 10% FCS.

Preparation of cell extracts and immunoprecipitation
Immunoprecipitation from the cell lysates was performed as described previously (19). In brief, cells were washed three times with ice-cold PBS, and lysed in TCSD buffer (50mM Tris HCl pH 7.4, 1% CHAPS, 300mM NaCl, 1mM dithiothreitol (DTT), 10mM NaF, 1mM PMSF, 1µg/ml leupeptin, 1µg/ml aprotinin) on ice for 1 hour, with occasional shaking. The lysate was then centrifuged at 10,000g at 4°C for 1 hour. Equal aliquots of the supernatant fractions were incubated overnight with appropriate antibodies and Protein A-Sepharose CL4B beads (Amersham Pharmacia Biotech) at 4°C. After this incubation period, sepharose beads were washed five times with TCSD buffer. Proteins bound on the beads were released by boiling them in the SDS sample buffer (20) for 5 min, then loaded on a polyacrylamide gel for electrophoresis.

**Synchronization of the cell cycle**

Synchronization of the cell cycle was achieved by double thymidine block (21) with some modifications. In brief, HeLa cells in the exponential growth phase were exposed to 2.5 mM thymidine in DMEM/ 5 % FCS for 12 hours and then incubated in fresh medium for 12 hours. Cells were once again exposed to 2.5 mM thymidine for 12 hours, after which the block was released by replacing the medium with fresh DMEM/ 5 % FCS. Flow cytometry was performed for analysis of cell cycle as previously described. (22)

**Preparation of the anti-MgcRacGAP antibodies**

To generate the anti-MgcRacGAP antibodies, a fusion protein between glutathione S transferase (GST) and C-terminus domain of MgcRacGAP was produced in E. Coli. For preparation of a polyclonal antibody against MgcRacGAP, C-terminal 275 amino acids was purified and injected into rabbits. The antibody was purified from whole serum using the C-terminus of MgcRacGAP on an affinity column.
Expression constructs and retrovirus vectors

A Flag-tagged MgcRacGAP was cloned into EcoR I and Not I sites of a mammalian expression vector pME 18S (pME-MgcRacGAP) (23). The deletion construct that defects the N-terminal myosin-like domain (ΔMyo-MgcRacGAP) was generated by PCR with a 5’ primer that contained an EcoR I site (5’-GAAAGAATTCCGAGAGATGCTCATGTGA-3’), and the 3’ primer that is located downstream of the BstXI site in MgcRacGAP (5’-TTCACCAACAGCTTGTTGACAT-3’). The resulting PCR fragment was digested with EcoR I and BstXI, and subcloned into pME-MgcRacGAP (pME-ΔMyoMgcRacGAP). A mutant MgcRacGAP lacking the cystein rich domain (pME-ΔCysMgcRacGAP) was generated by overlapping extension, using PCR. GTPase activating activity defective mutant (R386A*-MgcRacGAP) was generated by overlapping extension PCR mutagenesis. PCR was carried out using a high fidelity DNA polymerase Pyrobest (Takara). The PCR amplified sequence was confirmed by an automated sequencing using an ABI PRISM 310 Genetic analyzer (Perkin-Elmer).

A cDNA for MgcRacGAP was cloned into EcoRI – NotI sites of a retrovirus vector pMX-IRES-EGFP (24), upstream of IRES (internal reboosomal entry site) sequence so that both MgcRacGAP and EGFP were expressed from a single mRNA (pMX-MgcRacGAP-IRES-EGFP).

Retrovirus-mediated gene transfer

High titer retroviruses were produced from pMX-MgcRacGAP-IRES-EGFP, using a transient retrovirus packaging cell line PLAT-E (25), as described previously (26). We first established stable transfectants expressing the ecotropic viral receptor (27,28). For infection, HL-60 and Jurkat cells transduced with the ecotropic viral receptor and Ba/F3 cells (1×10⁵) were incubated with 10 ml of the retroviruses in the presence of hexadimethrine bromide (10 μg/ml)(SIGMA). Twenty-four hours after infection, cells were washed, and refed with growth medium. Two days after infection, the infected
cells were washed twice with PBS, suspended in PBS containing 1% BSA, and then sorted based on EGFP expression by a FACS Vantage (Becton Dickinson). The sorted cells were then expanded in a growth medium.

**Glutathione S-Transferase (GST) fusion proteins**

The myosin-like domain (Myo; amino acids 1-124), the cystein rich domain (Cys; amino acids 280-340), and three blocks conserved in RhoGAPs (GAPD; amino acids 345-620) were derived from MgcRacGAP and each domain was cloned into a pGEX-2T vector (Amersham Pharmacia Biotech). The transformed bacteria (DH5α) were grown, incubated with isopropyl-1-thio-β-D-galactopyranoside (IPTG) (1mM), lysed and the GST fusion proteins were purified according to the protocol of the manufacturer.
Results

Enforced expression of MgcRacGAP induced growth suppression and formation of multinucleated cells in hematopoietic cells.

Using retrovirus-mediated expression screening, we identified a full-length cDNA for MgcRacGAP in the anti-sense configuration based on the ability to inhibit IL-6 induced differentiation of M1 cells. Overexpression of MgcRacGAP induced macrophage differentiation in HL-60 (18). We also found that expression of MgcRacGAP partially inhibited proliferation in the cell line HL-60, M1, Ba/F3, and TF-1 (data not shown). Thus, MgcRacGAP seemed to regulate differentiation and proliferation of hematopoietic cells.

To determine the mechanisms by which MgcRacGAP regulates cell proliferation, HL-60, M1, Ba/F3, Jurkat and HeLa cells were transduced with a bicistronic retrovirus vector pMX-IRES-EGFP carrying a full-length cDNA for MgcRacGAP, where EGFP expression guarantees the expression of exogenous MgcRacGAP. EGFP-positive cells transduced with these viruses were sorted by FACS 2 days after infection. EGFP-positive cells transduced with a control pMX-IRES-EGFP vector were similarly sorted and served as a negative control.

Overexpression of MgcRacGAP retarded proliferation of all cell lines tested in this study. Unexpectedly, morphological analysis of Jurkat and Ba/F3 cells overexpressing MgcRacGAP detected a significant number of multinucleated giant cells (Fig. 1). A small number of multinucleated cells were also found in HL-60 and HeLa cells overexpressing this GAP (data not shown). These results suggested that MgcRacGAP was playing some roles in cytokinesis. To determine whether MgcRacGAP overexpression blocked the normal progression of mitosis, we performed cell cycle analysis in HL-60 overexpressing MgcRacGAP. However, no remarkable change was observed when compared with control cells, except for marginal increase of the cells in G2/M phase, which may have been caused by presence of multinucleated cells (data not...
Expression of MgcRacGAP is cell cycle-dependent

Because MgcRacGAP was implicated in cytokinesis, we investigated if the expression levels of MgcRacGAP change throughout the cell cycle. To this end, HeLa cells were synchronized at the G1/S transition by double thymidine block, and followed through to completion of mitosis. After release from the G1/S transition, the cells were analyzed for expression of MgcRacGAP mRNA. The DNA content at each time point was analyzed by flow cytometry (Fig. 2A). The mRNA levels for MgcRacGAP were gradually increased as the cells progressed through S phase, and reached the maximum at 9 to 12 hours after the release, corresponding to the G2/M phase (Fig. 2B). Thus expression of MgcRacGAP mRNA was cell cycle-dependent, and peaked during the G2/M phase. These observations, together with the findings that overexpression of MgcRacGAP induced formation of multinucleated cells, suggested that MgcRacGAP functions during mitosis, in particular during cytokinesis.

MgcRacGAP co-localized with the mitotic spindle and midbody in M phase

To further examine the function of this protein during the cell cycle, rabbit polyclonal antibodies against a recombinant protein corresponding to the C-terminal half of MgcRacGAP was prepared, as described in Materials and Methods. This antibody specifically recognized an 85kD protein (Fig. 3A).

To observe the subcellular distribution of the endogenous MgcRacGAP, HeLa cells were fixed, and MgcRacGAP was immunostained using the antibodies. In interphase, MgcRacGap was mainly localized in the nucleus although no Rho family proteins have been reported to localize in the nucleus. MgcRacGAP was also detected in the cytoplasm as a reticular pattern, along with microtubules (Fig. 3B, panel A).

MgcRacGAP spread throughout the cytoplasm in prometaphase and accumulated in the mitotic spindle in metaphase (Fig. 3B, panel B to C). In anaphase and telophase,
MgcRacGAP formed a distinct fine band extending across the midzone (Fig. 3B, panel D to E). As the cell progressed to cytokinesis, MgcRacGAP became more sharply concentrated in the midbody (Fig. 3B, panel F). To exclude the possibility that the antibodies we used in this study crossreacted with some protein other than MgcRacGAP, we introduced Flag-tagged MgcRacGAP, followed by immunodetection with an antibody against Flag (M2 monoclonal antibody). In this experiment, flag-tagged MgcRacGAP was also detected in the midbody of dividing cells, thus confirming that anti-MgcRacGAP antibodies specifically recognize MgcRacGAP in immunostaining (Fig. 4).

Expression of a myosin-like domain-defective mutant (ΔMyo-MgcRacGAP) or a GAP activity-defective mutant R386A*MgcRacGAP resulted in formation of multinucleated cells

MgcRacGAP contains three conserved domains, including an N-terminal myosin-like coiled-coil domain, a cystein-rich domain, and a C-terminal GAP-conserved domain. To determine which domain of MgcRacGAP was important for the control of cytokinesis, we constructed three mutants of MgcRacGAP; ΔMyo-MgcRacGAP lacking the myosin-like domain, ΔCys-MgcRacGAP lacking the cystein-rich domain, and R386A*MgcRacGAP with a GAP-inactivating mutation (Fig. 5A). All of these mutants were Flag-tagged at the C-terminus, and were introduced into a mammalian expression vector pME 18S (23). Expression of these mutant proteins was confirmed by transient expression in 293T cells, through lipofection. These mutant proteins as well as the wild type protein were expressed at similar levels in 293T cells (Fig. 5B).

Next we co-transfected pME-EGFP with a vector harboring the wild type or each mutants of MgcRacGAP into HeLa cells. pME 18S (23) was used as a vector control. The morphology of the EGFP positive cells was assessed 72 hours after transfection. When the wild type and mutant MgcRacGAPs were overexpressed in HeLa cells, some populations of the cells became multinuclear. Interestingly, expression of the ΔMyo-
MgcRacGAP and R386A*MgcRacGAP produced multinucleated cells more frequently (32% and 28% of the EGFP positive cells, respectively) than that of the wild type and ΔCys-MgcRacGAP did (10.5% and 8.5%, respectively) (Fig 5B). These data demonstrated that the GAP activity and the N-terminal myosin-like domain of MgcRacGAP were required for cytokinesis. It has to be noticed that this procedure may increase the frequency of the cells with lower expression levels of the transduced wild type or mutant MgcRacGAP, which may lead to underestimation of the frequencies of multinucleated cells after 72 hours.

**The N-terminal myosin-like domain is required for co-localization with microtubules.**

Analysis of deletion mutants indicated that the myosin-like domain and the GAP activity of MgcRacGAP were both required for completion of cytokinesis. Next, to identify the molecular mechanisms by which ΔMyo-MgcRacGAP and R386A*MgcRacGAP hampered cytokinesis, we expressed each mutant of MgcRacGAP in HeLa cells and immunostained using the anti-Flag antibody. Both mutant proteins entered the nucleus during interphase, as did the wild type of MgcRacGAP. However, unlike other mutants in cytokinesis, ΔMyo-MgcRacGAP was not detected in the midbody of dividing cells (Fig. 4). These results demonstrated that the N-terminal myosin-like coiled-coil domain was indispensable for localization to the midbody during cytokinesis, and also indicated that completion of cytokinesis requires the GAP activity of MgcRacGAP.

**MgcRacGAP associates with α-, β-, and γ-tubulin**

Immunohistochemical studies suggested that MgcRacGAP associates with microtubules and plays important roles in cytokinesis. To determine whether MgcRacGAP associates with microtubules in vivo, we performed co-immunoprecipitation using cell lysates from HeLa cells with the anti-MgcRacGAP
antibodies followed by Western blotting with monoclonal antibodies against α-, β-, and γ-tubulins. As shown in Fig. 6A, all tubulin isoforms were co-precipitated with MgcRacGAP from the cell lysates, indicating that MgcRacGAP associates with microtubules in vivo (Fig. 6A).

To confirm the association between MgcRacGAP and α-, β-, and γ-tubulins, reciprocal immunoprecipitations were also performed. MgcRacGAP was detected in the precipitates of α-, β-, and γ-tubulins (Fig. 6B). In these experiments, immunoprecipitation experiments were carried out with a lysis buffer containing detergent and high concentrations of salt as described in Materials and Methods to avoid non-specific binding between MgcRacGAP and tubulins as much as possible.

**The N-terminal myosin-like domain of MgcRacGAP is required for its binding to tubulins**

To determine which domain of MgcRacGAP is required for its association with tubulins, we transiently transfected deletion mutants of MgcRacGAP that were Flag tagged (Fig. 4A) into 293T cells. Two days after transfection, the cell lysates were prepared and expression of each mutant was examined with the anti-Flag antibody (Fig. 7A upper panel). Immunoprecipitation was performed with the monoclonal antibody against α-tubulin. The wild type MgcRacGAP, ΔCys-MgcRacGAP and R386A*MgcRacGAP were detected in α-tubulin immunoprecipitates, whereas no ΔMyo-MgcRacGAP mutant was detected (Fig. 7A lower panel), demonstrating that MgcRacGAP binds tubulin through its myosin like domain. Duplicate immunoprecipitation was performed with antibodies against β-, γ-tubulin, with essentially the same results (data not shown). To further confirm this result, GST-fusion proteins containing each domains of MgcRacGAP were incubated with HeLa cell lysates, and the precipitates were analyzed for the presence of α-, β-, and γ-tubulins. As shown in Fig. 7B, all tubulin isoforms were precipitated by GST-Myo (Myosin like domain), but not by the other domain constructs. These results indicated that the N-
terminal myosin-like domain mediates the association of MgcRacGAP and tubulin.
Discussion

In this study, we have shown that MgcRacGAP plays critical roles in cytokinesis, which is mediated by its localization to the central spindle and the midbody through binding to microtubules in the late M phase. Originally, we cloned the cDNA for MgcRacGAP through functional expression cloning which inhibited IL-6 induced macrophage differentiation of M1 cells, when expressed in the anti-sense configuration (18). On the other hand, overexpression of MgcRacGAP retarded cell growth and induced a significant number of multinucleated cells in all cell lines tested including HL-60, M1, Ba/F3, Jurkat and HeLa cells (Fig. 1 and data not shown). These data implicated that MgcRacGAP has some roles in cytokinesis. In addition expression levels of the mRNA for MgcRacGAP increased by 3 to 4 fold in the G2/M phase (Fig. 2). These results are consistent with previous study of Wooltorton et al. (29). They showed that expression of MgcRacGAP in 3T3-L1 cells and C2C12 cells were growth-regulated. We thus assumed from these findings that MgcRacGAP controls cell proliferation through regulating the progression of the G2/M phase. However, how MgcRacGAP retarded cell proliferation remains to be determined.

Immunohistochemical studies revealed that in interphase, MgcRacGAP localized in the nucleus and in the cytoplasm along with microtubules, then redistributed to the central spindle in anaphase and to the midbody in late telophase (Fig. 3B). This distribution implied the possibility that MgcRacGAP interact with microtubules. In fact, we found by co-immunoprecipitation experiments that MgcRacGAP was associated with α- and β-tubulin. Immunostaining showed that the N-terminal myosin-like domain of MgcRacGAP was required for its localization to the midbody of dividing cells (Fig. 5). Immunoprecipitation studies revealed that the myosin-like domain is responsible for its association with tubulin (Fig. 7). The myosin-like domain of MgcRacGAP contains a coiled-coil structure (30), and tubulin also dose. Therefore, it is predicted that interaction of these two coiled-coil domains mediates binding of MgcRacGAP to tubulins. Moreover, the overexpression of ΔMyo-MgcRacGAP led to failure of
cytokinesis, which was demonstrated by a markedly increased number of multinucleated cells. However, transfection of the wild type MgcRacGAP also causes abortive cytokinesis albeit at a low level. We assume from these data that expression of this protein at physiological levels and/or changes of the expression level through the cell cycle are important for normal progression of cytokinesis.

It was previously reported that tubulin mediates nuclear translocation of the glucocorticoid receptor and the vitamin D receptor (31,32). It was also proposed that c-Myc translocates from the nucleus to the cytoplasm along with tubulin (33). It is therefore possible that tubulin binding allows MgcRacGAP to use the microtubule network for its translocation in the mitotic phase. Taken together MgcRacGAP apparently plays important roles in cytokinesis through interaction with and movement on microtubules. Tubulin and microtubules have been shown to interact with regulatory components of the cell cycle apparatus as well as cellular oncogene products. For example, tubulin was shown to associate with neurofibromin to inhibit the Ras GAP activity (34). In an analogy, the association of tubulin with MgcRacGAP may regulate the GAP activity for Rac and Cdc42 in the mitotic structure and then control the cytokinesis.

The association of MgcRacGAP with α- and β- tubulin prompted us to examine whether MgcRacGAP also binds to γ-tubulin, and we found that MgcRacGAP associated with γ-tubulin as well. γ-tubulin has been identified in the centrosomes (35) and the midbody (36) during the mitotic phase. Observation with a confocal laser scanning microscope and in situ extraction immunostaining revealed that MgcRacGAP also localized to centrosomes in the mitotic phase (unpublished results). Disruption of γ-tubulin during anaphase by injection of either antibodies or anti-sense RNA led to failure of midbody formation and consequently a failure of cytokinesis (36,37), indicating that the nucleation of new microtubules may be important for the formation or maintenance of the midbody. Whether or not MgcRacGAP has a role in the microtubule nucleation or midbody formation and/or maintenance remains to be
determined.

During the preparation of this manuscript, Jantsch-Plunger et al. published a manuscript which contains a genetic, biochemical and cell biological analysis of the CYK-4 protein from C elegans (38). Many features of MgcRacGAP that we present here using the mammalian system are similar to those previously reported in the nematode system. CYK-4 localizes to the central spindle and persists at cell division remnants. They have also shown that central spindle assembly was defective in the cyk-4 (t1689ts) allele that carries a mutation at the N-terminus. This is probably because that the N-terminal myosin-like domain is required for its correct localization and association with microtubules and is indispensable for the cytokinesis in an analogy with MgcRacGAP shown in the present paper. Jantsch-Plunger et al. speculated that RhoA is the target of CYK-4 GAP activity for cytokinesis. In fact, RhoA was reported to be essential for cytokinesis and to be localized to midbody (10,39-41). CYK-4 activates GTP hydrolysis not only by Rac1 and Cdc42 but also by RhoA, albeit to a weaker extent with a much slower time course. Toure et al. also showed that MgcRacGAP had 30 times less GAP activity on Rho compared to Rac and Cdc42 (17). However, in contrast to these reports, we did not detect any GAP activity of MgcRacGAP on RhoA (18). Thus, although our data showed that the GAP activity of MgcRacGAP is required for cytokinesis (Fig. 5B), it is not clear at present which small G protein is a target of MgcRacGAP in the central spindle and the midbody. While in lower eukaryotes, a human homologue of Rac has been shown to be involved in cytokinesis (6,42). However, there is no report that implicated Rac in cytokinesis in mammals. Concerning Cdc42, expression of a constitutive active Cdc42 in a xenopus egg and a human cell line induced formation of multinucleated cells (5,41). These data indicated that Cdc42 is involved in cytokinesis. However, there is no data showing that Cdc42 localizes to the mitotic spindle or the midbody in mammals. Although further studies will be required to define the target of MgcRacGAP in the mitotic spindle, one possible explanation is that there is an unknown GTPase, which is involved in
cytokinesis and localized to the central spindle and the midbody. It is also possible that MgcRacGAP changes its specificity toward Rho with some modification when it localizes to the central spindle and the midbody. In fact, we have some preliminary data suggesting that MgcRacGAP is phosphorylated in the midbody. Recently various midbody-localized protein kinases have been isolated in vertebrate cells, including AIM-1 (43), AUR 1 (44), citron kinase (10), ERK/MKK (45), and Plk (46). Some of these kinases may function as regulators of MgcRacGAP localization and/or GAP activities, and these possibilities are now under investigation.

In summary, we have shown that MgcRacGAP associates with microtubules and plays important roles in cytokinesis. Recently there have been several notable developments in the field of cytokinesis. However, a molecular understanding of cytokinesis still remains elusive. We believe that further work on MgcRacGAP will provide some new insights into cytokinesis. But, how overexpression of MgcRacGAP induced macrophage differentiation of HL-60 cells, and how anti-sense MgcRacGAP inhibited IL-6-induced differentiation of M1 cells is still open to question.
References

32. Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C.,


Acknowledgements

We thank A. Kaneko for excellent sorting on FACS and M. Ohara for providing language assistance.
Footnotes

The Department of Hematopoietic factors was supported by Chugai Pharmaceutical Company Ltd. This work was also supported by grants from the Ministry of Education, Science and Culture of Japan and the Ministry of Health and Welfare of Japan.

The abbreviation used are: GEF, guanine nucleotide exchange factor; GAP, GTPase activating protein; FITC, fluorescein-isothiocyanate; CHAPS, (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate); PMSF, phenylmethylsulfonyl fluoride; EGFP, enhanced green fluorescence protein;
Figure legends

Fig. 1 Overexpression of MgcRacGAP induced multinucleated cells.
Ba/F3 and HL-60 cells were transduced with a retrovirus vector pMX-MgcRacGAP-IRES-EGFP. Ba/F3 and HL-60 cells were centrifuged onto glass slides and stained with May-Grünwald Giemsa solution.

Fig. 2 Cell cycle-dependent changes in the expression levels of the mRNA for MgcRacGAP.
Exponentially growing HeLa cells were synchronized at the G1/S transition by double thymidine block. Cells were harvested at indicated times for FACS analysis and RNA isolation.
(A) DNA contents of synchronized HeLa cells
FACS analysis was performed on HeLa cells harvested at 0, 1.5, 3, 6, 9, and 12 hours after release.
(B) Northern blot of synchronized HeLa cells probed with a \(^{32}\)P-labeled MgcRacGAP cDNA. Equal amounts of total RNA (20 µg) were loaded in each lane and probed with a \(^{32}\)P-labeled MgcRacGAP cDNA. Reprobing was carried out using \(^{32}\)P-labeled human G3PDH cDNA as a loading control. The mRNA levels were quantified by radioactivity using a Fujix BAS2000 bioimage analyzer (Fuji Photo Film Co., Tokyo, Japan). Signal intensities of MgcRacGAP were normalized by the GAPDH mRNA level, and were shown in the lower column.

Fig. 3 MgcRacGAP localizes to mitotic structures.
(A) Specificity of the affinity-purified anti-MgcRacGAP antibody.
Total cell lysates of HeLa cells (lane 1) and purified GST-MgcRacGAP C-terminus fusion protein (lane 2) were separated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with anti-MgcRacGAP antibody.
(B) MgcRacGAP localizes to the central spindle and the midzone in cytokinesis. HeLa cells at various stages of mitosis were stained with the anti-MgcRacGAP polyclonal antibody (left column), anti α-tubulin mAb (middle column), and DAPI for DNA staining (right column); (A) interphase, (B) prometaphase, (C) metaphase, (D) anaphase, (E) telophase, (F) cytokinesis.

Fig. 4 Localization of the wild type and each mutant forms of MgcRacGAP in HeLa cell.

HeLa cells were transfected with cDNA encoding Flag-tagged wild type and mutant forms of MgcRacGAP. After 48 hours, cells were stained with anti α-tubulin monoclonal antibody (red) and rabbit anti Flag polyclonal antibody (green).

Fig. 5 Effects of expression of the wild type and mutants MgcRacGAP on cytokinesis

(A) The structures of the wild type, mutant R386A*MgcRacGAP and deletion mutants of MgcRacGAP.

(B) Expression of the wild type and the mutant forms of MgcRacGAP

The 293T cells were transiently transfected with Flag-tagged MgcRacGAP constructs as indicated. Forty-eight hours after transfection, cells were collected and lysed by TCSD buffer. Total cell lysates were subjected to immunoblotting with a rabbit anti Flag polyclonal antibody.

(C) Expression of ΔMyo-MgcRacGAP and R386A*MgcRacGAP induced abortive cytokinesis.

MgcRacGAP (wild type), ΔMyo-MgcRacGAP, ΔCys-MgcRacGAP, R386A*MgcRacGAP, or pME18S as a vector control was co-transfected with a reporter construct pME EGFP into HeLa cells. EGFP-expressing cells were visualized by green fluorescence (upper column). DNA was stained with DAPI (middle column). GFP expressing multinucleated cells were counted under an immunofluorescence microscopy 72 hr after transfection. Data represent average of three independent experiments.
Fig. 6 Association of MgcRacGAP with α-, β- and γ-tubulins in vivo

(A) Co-immunoprecipitation of tubulins with MgcRacGAP

Cell extracts of interphase and mitotic HeLa cells synchronized with double thymidine block were subjected to immunoprecipitation with an anti MgcRacGAP antibody. The immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed by monoclonal antibodies against α-, β-, and γ-tubulins. Reprobing was carried out by antibodies against MgcRacGAP.

(B) Co-immunoprecipitation of MgcRacGAP with tubulins

Immunoprecipitations were performed with anti α-, β-, and γ-tubulin mAbs and precipitates were separated by SDS-PAGE, followed by blotting with an anti MgcRacGAP antibody.

Fig. 7 N-terminal myosin like domain mediates the association with microtubules.

(A) N-terminal myosin-like domain was required for its binding to tubulins.

293T cells were transiently transfected with Flag-tagged MgcRacGAP constructs as indicated. Forty-eight hours after transfection, cells were collected and lysed by TCSD buffer. Total cell lysates (upper panel) or immunoprecipitates with anti α-tubulin mAb from transfected cells (lower panel) were subjected to immunoblotting with a rabbit anti Flag polyclonal antibody

(B) GST pull down assay of distinct domains of MgcRacGAP

The GST fusion proteins containing each of three distinct domains of MgcRacGAP were incubated for 4 hours at 4°C with HeLa cell lysates and the precipitated proteins were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membrane was probed by α-, β-, and γ-tubulin mAbs.
Fig. 2

A

<table>
<thead>
<tr>
<th>0 hr</th>
<th>1.5 hrs</th>
<th>3 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6 hrs</th>
<th>9 hrs</th>
<th>12 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>1.5</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MgcRacGAP

G3PDH

Relative expression level vs. hours after release

Relative expression level

0 1.5 3 6 9 12 (hours after release)
Fig. 4

anti-Flag

Wild type

ΔMyo

ΔCys

R386A*
Fig. 5

A

wild type

\[ \text{myosin like coiled-coil domain} \]
\[ \text{Cysteine rich domain} \]
\[ \text{Three blocks conserved in Rho GAP} \]
\[ \text{Flag tag} \]

\[ \Delta \text{Myo MgcRacGAP} \]

\[ \Delta \text{Cys MgcRacGAP} \]

\[ \text{R386A* MgcRacGAP} \]

B

IB: anti Flag

--- total lysate ---

(Mr) K

--- 97.4 ---

--- 68 ---

wild type

\[ \Delta \text{Myo MgcRacGAP} \]

\[ \Delta \text{Cys MgcRacGAP} \]

\[ \text{R386A* MgcRacGAP} \]

vector
Fig. 5C

ΔMyo-MgcRacGAP  R386A*MgcRacGAP

GFP

DAPI

merge
% OF MULTINUCLEATED CELLS

vector cont.

Wild type

ΔMyo

ΔCys

R386A*
Fig. 6

A

**IP:** anti-MgcRacGAP cont. anti-MgcRacGAP cont. anti-MgcRacGAP cont.

**IB:** ---α-tubulin ---β-tubulin ---γ-tubulin ---

reblot: ___________ anti MgcRacGAP ___________

B

**IP:** α-tubulin cont. β-tubulin cont. γ-tubulin cont.

**IB:** ___________ anti MgcRacGAP ___________
Fig. 7

A

IP:  — anti-α tubulin —
IB:  — anti Flag —

wild type
ΔMyo MgcRacGAP
ΔCys MgcRacGAP
R336A MgcRacGAP
vector

B

IB: anti-α tubulin
(Mr) K 68

IB: anti-β tubulin
43

IB: anti-γ tubulin
43

GST GST-Myo GST-Cys GST GAPD
MgcRacGAP is involved in cytokinesis through associating with mitotic spindle and midbody
Koichi Hirose, Toshiyuki Kawashima, Itsuo Iwamoto, Tetsuya Nosaka and Toshio Kitamura

J. Biol. Chem. published online November 20, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007252200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2000/11/20/jbc.M007252200.citation.full.html#ref-list-1