Although many proteins have been shown to participate in mitotic events, including cytokinesis, their specific roles and interactions remain unclear. A novel interaction of proteins is demonstrated in this report. Yeast two-hybrid screening using PRC1 (protein-regulating cytokinesis 1) cDNA, a human mitotic spindle-associated cyclin-dependent kinase (CDK) substrate, which is involved in cytokinesis, as bait was performed. Data show that the PRC1 bait bound to MgcRacGAP, which is a GTPase-activating protein (GAP) for the Rho family GTPases also involved in cytokinesis. In addition, the two proteins showed similar localization during the M phase. PRC1 was shown to bind to the COOH-terminal GAP-conserved domain of MgcRacGAP and to inhibit its GAP activity toward Cdc42. This binding and/or inhibition of MgcRacGAP GAP activity was found to depend on further binding of PRC1 to the basic region (125–285 amino acids) of MgcRacGAP. Furthermore, the basic region was phosphorylated with Aurora B kinase, and this phosphorylation prevented the inhibition of GAP activity by PRC1. Cells overexpressing a phosphorylation mimic mutant of MgcRacGAP exhibited an abnormality of spindle morphology in the metaphase. Cdc42 showed high activity and was localized to the mitotic spindles and centrosomes during the metaphase. We propose that PRC1 down-regulates the GAP activity of MgcRacGAP during the metaphase and thereby contributes to the correct formation of the spindle.

Cytoskeletons, such as those comprising actin, microtubules, and intermediate filaments, help shape different cells, modulate cell motility, and organize various intracellular components in all eukaryotes. During the M phase, the dynamics of these cytoskeletons are one of the crucial factors for cells for accurate mitosis and cytokinesis. For example, the actin cytoskeleton, with myosin II, transiently constitutes the device called the contractile ring that divides the cytoplasm between two daughter cells. It has also been shown that the microtubule cytoskeleton forms large arrays termed mitotic spindles that segregate condensed chromosomes and forms the central spindle during the anaphase for successful cytokinesis.

Mammalian Rho family small GTPases (including RhoA, Rac1, and Cdc42) are required for many actin cytoskeleton-dependent cellular processes, including cytokinesis, and RhoA has been suggested to play a crucial role in the regulation of cell cleavage in mammalian cells (3–5). Active RhoA activates downstream effectors, such as ROCK/BOK/Rho kinase, citron kinase, and mDia/formin, which then promote actin polymerization and F-actin organization, stimulate myosin II activity, and promote the assembly and contraction of actomyosin filaments (3, 5–11). Based on the results of studies involving a constitutively active mutant of Cdc42, it has also been suggested that Cdc42 is involved in the M phase (12). However, mitotic events in which Cdc42 is involved have not yet been determined. Recent data suggest that Rho family GTPases not only control actin cytoskeleton remodeling but also regulate microtubule cytoskeleton stabilization (13–15), indicating that Cdc42 participates in the regulation of microtubule dynamics during mitosis.

Rho family GTPases cycle between GDP-bound forms (inactive) and GTP-bound forms (active), and upstream factors that regulate this cycle comprise two classes of proteins, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (16). These regulatory factors have been reported to be proteins involved in cytokinesis, ECT2, acting as a RhoGEF, and MgcRacGAP, acting as a RhoGAP. These proteins are localized to the midbody during cytokinesis and are therefore involved in this process (4, 17–19). Probably, the ECT2 GEF activity is required for Rho GTPase activation and ring formation, whereas the MgcRacGAP GAP activity induces Rho GTPase inactivation and ring disappearance. Moreover, studies have shown that the ECT2 GEF activity is regulated through its phosphorylation during mitosis, which results in an increase in activity, and by the NH2-terminal region of ECT2 itself, which acts as an inhibitor (4, 17). In regards to MgcRacGAP, although its GAP activity toward RhoA may be essential for completion of cytokinesis, it has been reported that the in vitro targets of MgcRacGAP are Cdc42 and Rac1 and that MgcRacGAP exhibited no (or very weak) GAP activity toward RhoA (20, 21). However, a recent study (22) was found to show that Aurora B kinase, which is a chromosomal passenger protein, phosphorylates the COOH-terminal GAP-conserved domain of MgcRacGAP at the Ser-387 residue in the midbody. In turn MgcRacGAP was seen to act as a RhoGAP (22). However, how GAP activity of MgcRacGAP toward Cdc42 or Rac1 is regulated while cells are undergoing mitosis remains unclear.

PRC1 (protein-regulating cytokinesis 1), which is a human regulator of mitotic spindle formation, has been shown to down-regulate the GAP activity of MgcRacGAP during the metaphase. This suggests that PRC1 may play a role in the regulation of mitotic spindle formation.
mitotic spindle-associated CDK (cyclin-dependent kinase) substrate, has also been known to be involved in central spindle formation and cytokinesis like MgcRacGAP and Aurora B (2, 23–27). Injection of anti-PRC1 antibodies (23, 24) or reduction of PRC1 protein level by means of small interference RNA (24) have been shown to prevent cell cleavage due to central spindle disruption, indicating the importance of PRC1 in cytokinesis. However, PRC1 is one or more mechanisms during early mitosis remain unknown (e.g., the significance of PRC1 being localized to the mitotic spindle in the metaphase). In this regard, on comparison of the cellular localization of PRC1 and MgcRacGAP, these proteins showed similar patterns during the M phase, including the metaphase (18, 23, 24); accordingly, there is the possibility that these two proteins associate with each other during early mitosis. Here, we report that MgcRacGAP was identified as a protein that interacts with PRC1 on yeast two-hybrid screening, as well as a novel regulatory mechanism of MgcRacGAP GAP activity by PRC1.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening System—Bait for two-hybrid screening was obtained by cloning of the complete coding sequence of human PRC1 by RT-PCR and subcloning into the pGBT9 vector, which yielded a fusion with the GALA DNA binding domain. A mouse lymphoma cDNA library, cloned into pACT (Clontech), was screened for PRC1-binding proteins in a S. cerevisiae reporter strain, Y190. Yeast cultures and two-hybrid procedures were carried out according to the manufacturer’s instructions (Clontech). Library plasmids from transformed yeast colonies were recovered using Escherichia coli HB101 as the recipient strain and selected on M9 minimal medium. cDNA inserts from clones interacting specifically with PRC1 were sequenced with an ABI PRISM 3100 automated sequencer, and a BLAST algorithm was used to search for homology with known GenBank™ sequences.

Expression and Purification of Recombinant Proteins—Human PRC1, MgcRacGAP, Cdc42, and Aurora B cDNAs were obtained by reverse transcription-PCR. GST fusion and hexa-His fusion proteins were expressed in a baculovirus expression system (Life Technologies) and purified using glutathione-Sepharose 4B (Amersham Biosciences) and TALONTM Metal Affinity Resin (Clontech), respectively. GST-Cdc42 was expressed in E. coli (DH5α) using a pGEX vector (Amersham Biosciences). The deletion constructs of MgcRacGAP (ΔMyo-, ΔCys-, and ΔGAP) were prepared by PCR as described previously (21) and expressed as GST fusion proteins in Sf9 cells using a baculovirus expression system, ΔB-R-MgcRacGAP was prepared by PvuII digestion of MgcRacGAP cDNA, separation of the fragments, and self-igation of the large fragment. The MgcRacGAP GAP domain (GST-GAP); C-terminal 275 amino acids) was constructed by PCR and expressed in E. coli (DH5α) using the pGEX vector. The kinase-deficient mutant Aurora B (K106M) and Ala and Asp mutants of MgcRacGAP were constructed by PCR mutation. The MgcRacGAP basic regions (GST-B.R.) were prepared by PvuII digestion of MgcRacGAP cDNA and separation of the fragments, and expressed in E. coli (BL21(DE3)pLys) using the pGEX vector.

Cell Culture, Cell Cycle Synchronization, and Transfection—HeLa S3 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn bovine serum. HeLa S3 cells were synchronized in the S phase by a double-thymidine block method, as described previously (4). To arrest cells in the prometaphase, nocodazole (30 ng/ml, final concentration) was added 6 h after the release of cells from the double-thymidine block, and mitotic cells were collected by shake-off procedure 6 h later. The collected cells were then released from the nucodazole arrest into a fresh medium. At 0, 45, 90, and 180 min after release, cells were harvested and subjected to analyses. In indicated experiments (Figs. 1, 2, 5, and 6), cells were transfected with each plasmid using LipofectAMINE™ 2000 (Invitrogen) during the second thymidine block.

Immunofluorescence Microscopy—Synchronized HeLa S3 cells were immunostained with monoclonal anti-α-tubulin (Sigma; 1/500 dilution), polyclonal anti-PRC1 (Santa Cruz Biotechnology; 1/100 dilution), polyclonal anti-pericentrin (BA1020; 1/100 dilution), or monoclonal anti-Cdc42 (Santa Cruz Biotechnology; 1/100 dilution). Alexa FluorTM 594-labeled goat anti-mouse or anti-rabbit IgG antibodies (Molecular Probes; 1/100 dilution), or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibodies (Dako; 1/100 dilution) were used as secondary antibodies. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Samples were observed under a fluorescence microscope (Olympus).

In Vitro Binding Assays—The purified GST wild-type or mutant MgcRacGAPs were incubated for 20 min at 30 °C with purified His-PRC1 in 200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, and 1 mM EGTA in a final volume of 50 μl, and then pulled down using glutathione-Sepharose 4B (Amersham Biosciences). The resin was washed four times with the above buffer containing 0.2 mM PMSF, 2 mM DTT, and 0.1% Triton X-100, and the retained proteins were eluted with an SDS-PAGE sample buffer then subjected to SDS-PAGE. Western blotting was performed with monoclonal anti-penta-His antibodies (Qiagen), horseradish peroxidase-conjugated anti-mouse IgG antibodies (Dako), and ECL reagents (Amersham Biosciences). In some experiments, the phosphorylated form of a PRC1/MgcRacGAP, the phosphorylation reaction was carried out for 30 min at 30 °C with the purified His wild-type or kinase-deficient Aurora B (K106M) in 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, and 1 mM ATP, in a final volume of 50 μl.

Immunoprecipitation—Synchronized HeLa S3 cells were lysed by incubation for 30 min at 4 °C in 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 10 mM NaF, 0.2 mM DTT, 1× Protease inhibitor mixture (Roche Applied Science), and 1% Nonidet P-40. The lysates were diluted with an equal volume of the same buffer without NaCl and then incubated overnight at 4 °C with polyclonal anti-PRC1 antibodies (Santa Cruz Biotechnology) or normal rabbit IgG (Santa Cruz Biotechnology). Samples were centrifuged at 9,000 × g for 10 min at 4 °C, and then the supernatants were further incubated for 4 h at 4 °C with Protein A-Sepharose CL-4B (Amersham Biosciences). The immunoprecipitates were washed twice with 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 1 mM EGTA, 0.2 mM PMSF, 2 mM DTT, and 0.1% Nonidet P-40, and the retained proteins were eluted with an SDS-PAGE sample buffer. Eluted proteins were subjected to SDS-PAGE and Western blotting, probing sequence- and cell-cycle-regulated by Western blotting, probing sequence- and polyvalconal anti-FLAG (Sigma) and polyclonal anti-PRC1 antibodies.

In Vitro GTP Hydrolysis Assays—GTPase activity was measured by means of a nitrocellulose filtration assay, as described previously (28). Two micrograms of GST-Cdc42 were incubated for 10 min at 30 °C with 10−4 M MgCl₂, 10−3 M GTPγS, and 0.01% Detergent (Sigma) in 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM DTT, and 40 μg/ml of bovine serum albumin, in a total volume of 50 μl, and the exchange reaction was stopped with the addition of MgCl₂ (10 mM, final concentration). An 8-μl sample of the GST-Cdc42 (γ−32P)GTP complex was added to 12 μl of an exchange buffer (1 mM non-labeled GTP, 20 mM Tris-HCl, pH 7.5, 25 mM NaCl, 10 mM MgCl₂, 1 mM DTT, and 40 μg/ml bovine serum albumin) containing 50 or 100 μM purified GST-MgcRacGAP GAP domain (GST-GAPD), GST-MgcRacGAP wild-type or a mutant (ΔB.R.- or 5D), or Aurora B-phosphorylated GST-MgcRacGAP alone or together with several concentrations of GST-PRC1. Then the mixtures were incubated for 3 min at 25 °C. Samples (3 μl vol.) were then removed and filtered through nitrocellulose filters (Schleicher and Schuell), which were washed twice with an ice-cold buffer (1 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10 mM MgCl₂), and the remaining amounts of γ−32P,GTP on the filters were measured by scintillation counting.

In Vitro Phosphorylation Assays—The purified GST wild-type or mutant MgcRacGAPs were incubated for 30 min at 30 °C with purified GST-Aurora B in 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 4.5 mM 2-mercaptoethanol, 0.25 μCi of γ−32P,ATP (Amersham Biosciences), and 1 mM non-labeled ATP, in a final volume of 50 μl. The reactions were then stopped with the addition of an SDS-PAGE sample buffer. Protein samples were separated by SDS-PAGE and phosphate imaged on autoradiography.
Results

MgcRacGAP Is a Novel Binding Partner of PRC1—To identify the proteins binding to PRC1, we screened a mouse lymphoma cDNA library using a yeast two-hybrid system with the full-length human PRC1 coding sequence as bait. Among the $1.8 \times 10^6$ clones screened, twelve positive ones were identified, three of which were coded for MgcRacGAP, a GTPase-activating protein (GAP) for Rho family GTPases (20, 21). Because MgcRacGAP, like PRC1, has been reported to be involved in cytokinesis (18), we studied its interaction with PRC1 in detail.

First, we examined the cellular localization of endogenous PRC1 and GFP-tagged MgcRacGAP by means of fluorescence microscopy in cells synchronized in the M phase (Fig. 1). Endogenous PRC1 could not be detected, because we failed to produce antibodies against human MgcRacGAP. However, there is no difference exists between the localization that this GFP fusion protein showed here and that of endogenous MgcRacGAP reported previously (18). Endogenous PRC1 and GFP-MgcRacGAP were localized in the nucleus during the interphase. During the metaphase, both proteins accumulated in the mitotic spindle, but during the anaphase and telophase, both were concentrated in the central region between the separating chromosomes. As the cells underwent cytokinesis, GFP-MgcRacGAP was concentrated in the midbody. According to previous studies, PRC1 should only be localized in the midbody (23, 24), but in our study, it was found in not only the midbody but also the intercellular bridge; this may have been due to differences in antibodies used. In any case, we found that these two proteins showed similar localizations during the M phase.

Next, full-length human PRC1 and MgcRacGAP were expressed as hexa-His or GST fusion proteins in a baculovirus expression system and GST pull-down assays were performed. As shown in Fig. 2A, His-PRC1 was co-precipitated with GST-MgcRacGAP (left panel) and His-MgcRacGAP with GST-PRC1 (right panel). To determine whether or not these proteins interacted in cells, HeLa S3 cells were transfected with mammalian expression plasmids encoding 3×FLAG-MgcRacGAP and were synchronized in the M phase. The immunoprecipitates from the transfected cell lysates using anti-PRC1 polyclonal antibodies were examined by Western blotting. The anti-PRC1 antibodies specifically co-immunoprecipitated both endogenous PRC1 and 3×FLAG-MgcRacGAP, showing a specific association between the two proteins (Fig. 2B). These results demonstrate that PRC1 and MgcRacGAP associate both in vitro and in cells.

PRC1 Inhibits MgcRacGAP GAP Activity Toward Cdc42 in Vitro—The PRC1 binding region in MgcRacGAP was then studied. As shown in Fig. 3A, MgcRacGAP comprises three conserved domains (an N-terminal myosin-like coiled-coil domain, a cysteine-rich domain, and a C-terminal GAP-conserved domain) (21), and one basic amino acid region (amino acids 125–285). We constructed fusion proteins consisting of GST fused to wild-type MgcRacGAP or to four deletion mutants of MgcRacGAP lacking the myosin-like domain (∆Myo-MgcRacGAP), cysteine-rich domain (∆Cys-MgcRacGAP), GAP domain (∆GAP-MgcRacGAP), and basic region (∆B.R.-MgcRacGAP), respectively (Fig. 3A). The proteins were then immobilized on glutathione-Sepharose 4B beads, which were incubated with purified hexa-His-PRC1. After washing the resin, the bound proteins were eluted, and subjected to SDS gel electrophoresis and Western blotting with anti-penta-His antibodies. PRC1
bound equally well to the wild-type, and ΔMyo-, and ΔCys-MgcRacGAP, but only approximately half as well to ΔB.R.-MgcRacGAP and only weakly to ΔGAP-MgcRacGAP (Fig. 3B, top panel). These differences were not due to the amounts of GST fusion proteins used in the assay (Fig. 3B, bottom panel), indicating that PRC1 binds to both the GAP domain and the basic region of MgcRacGAP.

Since there has been no report on how MgcRacGAP activity toward Cdc42 is regulated during mitosis, our finding that PRC1 interacts with MgcRacGAP through its GAP domain suggests that PRC1 might be a regulator of MgcRacGAP activity. To test this, we performed in vitro GTP hydrolysis assays using recombinant human Cdc42. We prepared recombinant GST fusion proteins containing full-length MgcRacGAP, the MgcRacGAP GAP domain (GAPD), or deletion mutant ΔB.R.-MgcRacGAP. Because RhoA and Cdc42, but not Rac1, are involved in cytokinesis in mammalian cells (3–5, 12, 19), we focused on the GAP activity of MgcRacGAP toward Cdc42. We therefore carried out GAP activity assays using Cdc42 and GST-GAPD, GST-ΔB.R.-, or GST-full-length MgcRacGAP in the presence of different concentrations of purified GST-PRC1, and found that a 5-fold amount of PRC1 dramatically inhibited full-length MgcRacGAP GAP activity. On the other hand, in the presence of 10-fold PRC1, there was no effect on that of GAPD or ΔB.R.-MgcRacGAP (Fig. 3C).

Our findings involving His-PRC1 and GST-fused wild-type MgcRacGAP, or GAPD mutant GAPD (top panel). The bottom panel shows Coomassie Blue staining of the GST fusion protein samples used in the assay.

Phosphorylation of the Basic Region by Aurora B Prevents the Interaction between MgcRacGAP and PRC1—Because the MgcRacGAP GAP activity inhibited by PRC1 depended on the

![Fig. 3. Effect of PRC1 on the GAP activity of MgcRacGAP. A, schematic representation of wild-type MgcRacGAP and four deletion mutants. Each protein was expressed as a GST fusion protein. B, in vitro binding of His-PRC1 to the wild-type or mutant MgcRacGAP, using the system described in Fig. 2A (top panel). The bottom panel shows Coomassie Blue staining of the GST fusion protein samples used in the assay. C, the GAP activity of the GST-fused full-length MgcRacGAP, MgcRacGAP GAP domain (GAPD), or ΔB.R.-MgcRacGAP toward Cdc42 was measured in the presence or absence of the indicated concentrations of GST-PRC1. Each bar represents the mean ± S.D. of at least three independent experiments. D, in vitro binding of PRC1 to the MgcRacGAP GAP domain. The experiment was performed as described in Fig. 2A using His-PRC1 and GST-fused wild-type MgcRacGAP, or GAPD mutant GAPD (top panel). The bottom panel shows Coomassie Blue staining of the GST fusion protein samples used in the assay.](http://www.jbc.org/content/jbc/18/46/16397/F3)

![PRC1 Is an MgcRacGAP Inhibitor](http://www.jbc.org/content/jbc/18/46/16397/F3)
interaction of PRC1 with the basic region in vitro, we expected
that the mutant lacking the basic region (ΔB-R-MgcRacGAP)
would be an unregulatable mutant in regard to the GAP activ-
ity in cells. We then confirmed the cellular localization of GFP-
fused ΔB-R-MgcRacGAP in cells and found that the localiza-
tion of this mutant was distinct from that of the wild-type.
GFP-ΔB-R-MgcRacGAP spread out in the cytoplasm and did not
accumulate in the spindle from the metaphase to the an-
aphase. During cytokinesis, GFP-ΔB-R-MgcRacGAP was not
detected in the midbody of dividing cells like ΔMyo-MgcRac-
GAP (18) (data not shown). Because we had to avoid the effects
on M phase cells by wild-type MgcRacGAP and ΔB-R-MgcRac-
GAP due to the difference in their localizations, we tried to
generate other MgcRacGAP mutants showing normal localiza-
tion whose GAP activity is unregulatable accordingly.

A recent study showed that Aurora B kinase, which is a
chromosomal passenger protein, phosphorylates the GAP do-
main of MgcRacGAP at the Ser-387 residue, and that this
phosphorylation converts MgcRacGAP to RhoGAP (22). To
determine which regions of MgcRacGAP were phosphorylated
by Aurora B, we carried out in vitro phosphorylation of the con-
structs shown in Fig. 3A as substrates with GST fusion Aurora
B. The phosphorylation of ΔB-R-MgcRacGAP lacking the basic
region was hardly detectable (Fig. 4A), suggesting that Aurora
B strongly phosphorylates the basic region of MgcRacGAP, in
agreement with previous findings (22). This phosphorylation
was focused on, because the basic region was important for
the interaction between PRC1 and MgcRacGAP, and whether or
not this phosphorylation affected PRC1 binding to MgcRacGAP
was examined. GST-MgcRacGAP was incubated with either
the wild-type or kinase-dead mutant (K106M) of hexa-His-
tagged Aurora B, and then GST pull-down assays with hexa-
His-PRC1 were carried out. A lower rate of interaction with
PRC1 was observed for MgcRacGAP incubated with wild-type
Aurora B than its kinase-dead mutant (Fig. 4B). This lower rate
of interaction was not due to phosphorylation of the GAP
domain at the Ser-387 residue, but that of the basic region,
using GST fusion MgcRacGAP mutants with an Ala substitu-
tion at the Ser-387 residue (S387A) (Fig. 4C). Aurora B-phos-
phorylated MgcRacGAP-S387A also interacted with PRC1 less
than the wild-type, thus these data demonstrate that Aurora B
phosphorylation of the basic region prevents PRC1 interaction
with MgcRacGAP through its basic region in vitro.

As shown above, binding to the basic region of MgcRacGAP
was required for PRC1 to inhibit its GAP activity toward Cdc42
(Fig. 3C). To determine whether or not this MgcRacGAP phos-
phorylation of the basic region by Aurora B prevents the inhi-
bition of its GAP activity by PRC1, we carried out, as described
above, in vitro GTP hydrolysis assays with purified Aurora
B-phosphorylated GST-MgcRacGAP in the presence or absence
of PRC1. The results show that, as with GAPD and ΔB-R-
MgcRacGAP (Fig. 3C), the GAP activity of Aurora B-phos-
phorylated MgcRacGAP toward Cdc42 was not inhibited by a 5-fold
amount of PRC1, whereas the wild-type MgcRacGAP activity
was significantly decreased (Fig. 4D). Our findings indicate
that PRC1 is unable to inhibit Aurora B-phosphorylated
MgcRacGAP GAP activity toward Cdc42 in vitro and that the
phosphorylation of the basic region is useful for the generation
of GAP activity unregulatable mutants of MgcRacGAP.

PRC1 Down-regulates MgcRacGAP GAP Activity Toward
Cdc42 in Metaphase Cells and Is Required for Normal Spindle
Formation—In the next series of experiments, the one or more
Aurora B phosphorylation sites in the basic region of MgcRac-
GAP were identified. It has been reported that Aurora B is an
Arg-directed kinase and phosphorylates the Ser residue in the
RXS motif (29). Moreover, the Aurora B phosphorylation sites
of INCENP, which is also a chromosomal passenger protein,
and forms a complex called the AB1 complex with Aurora B and
survivin (26, 27, 30–33), are the three residues making up the
TSS motif (34). MgcRacGAP basic region fragment mutants
were then constructed, which involved combinatorial site-di-
rected mutagenesis of Ser or Thr residues, candidate phospha-
ylation sites, to Ala, as summarized in Fig. 5A. Following this,
GST-fused fragment mutants acting as Aurora B substrates in
vitro were examined. It was found that the 5A fragment in
which five Ser/Thr residues were substituted with Ala (S144A/
T145A/S185A/T186A/S187A) fully prevented phosphorylation
(Fig. 5B), confirming that Aurora B primarily phosphorylates
five Ser/Thr residues in the basic region of MgcRacGAP.

Next, a mutated full-length MgcRacGAP with five Ser/Thr
residues changed to Asp (MgcRacGAP-5D; S144D/T145D/
S185D/T186D/S187D) was constructed, and whether or not this
mutant could act as a phosphorylation mimic mutant was ex-
amined. After HeLa S3 cells transfected with 3×FLAG
tagged full-length wild-type MgcRacGAP, -5A, or -5D had been
synchronized in the M phase, we conducted immunoprecipita-
tion with each transfected cell lysate using anti-PRC1 poly-
clonal antibodies and examined the precipitate for endogenous
PRC1 and several 3×FLAG-MgcRacGAPs by Western blotting.
As expected, in contrast with the wild-type and 5A, endogenous
PRC1 bound weakly to MgcRacGAP-5D (Fig. 5C). Further-
more, judging from the results of in vitro binding assays in-
volving purified GST fusion proteins as to the wild-type
MgcRacGAP, -5A, or 5D-basic region, PRC1 indeed bound to
the basic region, and there was no difference in PRC1 binding
between the wild-type basic region and 5A basic region,
whereas interaction with the 5D basic region was never de-
tected (Fig. 5D). Moreover, to determine whether or not PRC1
inhibited the GAP activity of MgcRacGAP-5D toward Cdc42,
in vitro GTP hydrolysis assays were performed as described
above. As shown in Fig. 5E, the same result was obtained, i.e.
MgcRacGAP-5D GAP activity was not suppressed by a 5-fold
amount of PRC1, which is the effective concentration for inhib-
ing wild-type MgcRacGAP. From these results, it was dem-
onstrated that MgcRacGAP-5D acted as a phosphorylation
mimic mutant and that its GAP activity toward Cdc42 was
unregulatable by PRC1 in vitro.

In an attempt to determine the role of PRC1 in cells using
this MgcRacGAP-5D mutant, expression vector pEGFP carry-
ing MgcRacGAP-5D was constructed and its effects on HeLa S3
cells transfected with pEGFP-MgcRacGAP-5D by fluorescence
microscopy was observed. No difference between the wild-type
MgcRacGAP localization and that of the 5D mutant was found
(data not shown). However, it was notable that some cells,
which overexpressed MgcRacGAP-5D, exhibited an abnormal-
ity of spindle morphology in the metaphase (Fig. 6). There were
no aberrant metaphase spindles in the cells that expressed
wild-type MgcRacGAP (left panel). In contrast, in cells express-
ing 5D, i.e. unregulatable GAP activity mutant, bilateral met-
aphase spindles stretching from opposite poles were not ob-
served, and it seemed that spindles were catastrophic (right
panel). These results indirectly suggest that to maintain nor-
mal spindle morphology, PRC1 inhibits the GAP activity of
MgcRacGAP toward Cdc42 during the metaphase.

To confirm the changes in the level of GTP-bound Cdc42
during the M phase, GTP-Cdc42 pull-down assays were per-
formed using GST-fused CRIB (Cdc42/Rac1 interactive bind-
ing domain) of PAK1, an effector of Cdc42 and Rac1. For these
assays, HeLa S3 cells were prepared at 0, 45, 90, and 180 min
after nocodazole release. According to their cell morphology,
the cells collected at 0, 45, 90, and 180 min were at about the
prometaphase, metaphase, cytokinesis, and G1 phase, respec-
Fig. 4. Effect of phosphorylation by Aurora B on the interaction between PRC1 and MgcRacGAP. A, in vitro phosphorylation assaying of MgcRacGAP using the mutants shown in Fig. 3A. Each GST-fused MgcRacGAP trapped on glutathione-Sepharose beads was incubated with GST or GST-Aurora B in the presence of [γ-32P]ATP. The reactions were detected by autoradiography (top panel). The amounts of GST fusion protein used in the assay were compared by Coomassie Blue staining (bottom panel). B, binding of PRC1 and Aurora B-phosphorylated MgcRacGAP in vitro. GST-MgcRacGAP was first incubated with His-tagged wild-type or kinase-dead (K106M) Aurora B, then the assay was performed as described in Fig. 2A using these MgcRacGAPs and His-PRC1. C, a similar binding experiment was performed using the GST-fused wild-type MgcRacGAP or S387A mutant incubated with His-Aurora B in the presence or absence of ATP. D, effect of PRC1 on Aurora B-phosphorylated MgcRacGAP GAP activity. The GTPase activity of Cdc42 was measured by means of the nitrocellulose filtration assay as described in Fig. 3C. Each bar represents the mean ± S.D. of at least three independent experiments.
tively. Each cell lysate was then incubated with GST-CRIB, and the amount of GTP-Cdc42 was determined by Western blotting with anti-Cdc42 monoclonal antibodies. As can be seen in Fig. 7A, GTP-Cdc42 was at a low level at 0 min in the prometaphase cells, however, the level of GTP-Cdc42 increased at 45 min in the metaphase cells, indicating that Cdc42 was activated during the metaphase. Because there has been no report on the subcellular distribution of Cdc42 in metaphase cells, the localization pattern of Cdc42 was examined in the metaphase cells by immunofluorescence analysis with Cdc42 antibodies. Interestingly, the fluorescence of Cdc42 was observed on the mitotic spindles and centrosomes during the metaphase (Fig. 7B). Altogether, these data indicate that small GTPase Cdc42 is involved in the regulation of mitotic spindle formation during the metaphase and that PRC1 is required for the activation of Cdc42 through the down-regulation of MgcRacGAP activity.

DISCUSSION

The mitotic spindle-associated protein PRC1 is known to be essential for cytokinesis, and recent studies have shown that one of its functions, namely its ability to bind to and bundle microtubules, is required for central spindle formation during the anaphase (23, 24). A central spindle disturbance due to the absence of PRC1 causes a cytokinesis defect and thereby results in the production of multinucleated cells (24). Also, MgcRacGAP, which is a GAP for Rho family small GTPases, including RhoA, Rac1, and Cdc42, has been reported to be a crucial factor for cytokinesis (18, 19). When cells undergo cytokinesis, RhoA is locally activated at cleavage furrows and has been found to stimulate contractile ring formation and constriction (2–5). MgcRacGAP GAP activity is required for the completion of cytokinesis through ring disappearance subsequent to the inactivation of RhoA. In Caenorhabditis elegans, although embryos with mutations in CYK-4 (a MgcRacGAP orthologue) form cleavage furrows, these regress only after extensive ingestion (35). In mammalian cells, it has also been shown that a GAP activity-deficient mutant (S387A) or Aurora B phosphorylation site-defective mutant (S387A) of MgcRacGAP causes cytokinesis failure followed by cell polyploidy (18, 22). Thus, both PRC1 and MgcRacGAP are known as cytokinesis-related proteins; on the other hand, there has been no report on how the two proteins are involved in early mitotic events. For instance, why they both accumulate in the mitotic spindle during the metaphase (Fig. 1), or the significance of this co-localization. Likewise, it is not clear how MgcRacGAP GAP activity toward Cdc42 or Rac1 is regulated during the M
Because MgcRacGAP only acts as RhoGAP when its GAP domain is phosphorylated by Aurora B in the midbody (22), MgcRacGAP may not work for RhoA during early mitosis. On the other hand, MgcRacGAP normally shows its activity toward Cdc42 and Rac1 (20, 21); therefore, regulatory mechanisms for this activity are necessary. In this study, PRC1 was found to show that it inhibits the GAP activity of MgcRacGAP toward Cdc42, and this inhibition is important for the correct formation of the mitotic spindle during the metaphase; these findings revealed the roles of these proteins in early mitosis.

Cytoskeleton dynamics, such as those comprising actin, microtubules, and intermediate filaments, are essential for cells to progress accurately to the M phase. Small GTPases of the Rho family regulate the actin cytoskeleton, and RhoA and Cdc42 play particularly essential roles in cell division (3–5, 12). Moreover, recent studies suggest that, in addition to remodeling the actin cytoskeleton, Rho family GTPases also influence the organization and dynamics of microtubules (13–15). When cells overexpressed the MgcRacGAP mutant, whose GAP activity toward Cdc42 is not inhibited, in other words, there was an increased level of the inactivated state of Cdc42 in metaphase cells, mitotic spindle disturbance was observed (Fig. 6). Moreover, we also found that Cdc42 accumulated on the mitotic spindle, and its activity was increased during the metaphase (Fig. 7, A and B). These findings indirectly demonstrate that Cdc42 regulates the formation of the metaphase spindle. It is known that Ser/Thr kinase p65PAK is one of the common downstream targets of Cdc42 and Rac1 (11, 36). According to recent reports, p65PAK phosphorylates Ser-16 on stathmin (also termed Oncoprotein 18, p19, 19K, metablastin, and prosolin), and this phosphorylation is induced by the activation of Cdc42 and Rac1 in cells (13). Stathmin is a conserved protein that regulates microtubule dynamics, and its activity causes destabilization of growing microtubules (37). Interestingly, stathmin is inactivated by phosphorylation at four Ser residues (Ser-16, -25, -38, and -63) during mitosis; Ser-25 and Ser-38 are targets for CDKs, mainly Cdk1/Cyclin B, but the kinases that phosphorylate the other Ser residues (Ser-16 and Ser-63) have not been identified. Because these four phosphorylation sites are involved in the complete inactivation of stathmin and this phosphorylation is essential for the correct formation of the mitotic spindle during the metaphase (38–41), our findings suggest that p65PAK, which is activated by the active form of Cdc42, participates in this event. Thus, we can put forward the following hypothesis: there is the possibility that the down-regulation of MgcRacGAP activity toward Cdc42 by PRC1 contributes to the p65PAK-mediated inactivation of stathmin.

Moreover, in this report, the strong phosphorylation of the basic region of MgcRacGAP by Aurora B kinase was demonstrated, and this phosphorylation prevents the inhibition of MgcRacGAP GAP activity by PRC1 (Fig. 4). The Aurora B phosphorylation sites in the basic region of MgcRacGAP were also identified, i.e. five Ser/Thr residues (Ser-144, Thr-145, Ser-185, Thr-186, and Ser-187) (Fig. 5B). However, at this time, this phenomenon was used to generate the MgcRacGAP mutant. If these five Ser/Thr residues are indeed phosphorylated by Aurora B in cells, when does it occur and what role does it play? One possibility is as follows. Aurora B is one of the chromosomal passenger proteins, and its cellular localization exhibits the same features as those of other passenger proteins. During the metaphase, Aurora B is concentrated at centromeres, distinct from the localization of MgcRacGAP or PRC1. But upon transition to the anaphase, Aurora B is transferred to the spindle midzone called the central spindle, and is concentrated in the intercellular bridge during cytokinesis (42). From the anaphase to the telophase, Aurora B is co-localized with MgcRacGAP and PRC1 (data not shown), namely, if Aurora B phosphorylates the basic region of MgcRacGAP, it may occur from the anaphase. This possibility is supported by the recent finding that Aurora B phosphorylates MgcRacGAP in the mid-

![Image](http://www.jbc.org/)

**Fig. 6. Effect of overexpression of MgcRacGAP-5D on mitotic cells.** HeLa S3 cells were transfected with plasmids encoding GFP-tagged wild-type MgcRacGAP or -5D, and then synchronized in the M phase. Immunofluorescence staining of cells was carried out as described under “Experimental Procedures.” Green, GFP-wild-type MgcRacGAP or -5D; red, α-tubulin; blue, DAPI; and yellow, overlapping of GFP-wild-type MgcRacGAP or -5D with microtubules.
body (22). The phosphorylation of the MgcRacGAP basic region caused the release from the inhibition by PRC1 in vitro (Figs. 4D and 5E); therefore, MgcRacGAP may act for Cdc42 as a GAP during the anaphase or telophase. It has also been reported that a constitutively active mutant of Cdc42 causes cytokinesis arrest (12). There is the possibility that the phosphorylation of the basic region of MgcRacGAP by Aurora B during the anaphase or telophase is necessary for the actin polymerization of Cdc42 by MgcRacGAP for completion of cytokinesis. Although we did not obtain any cellular evidence of Aurora B phosphorylation in this study, the basic region of MgcRacGAP may play an important role in the regulatory mechanism for its GAP activity. Preceding metaphase, PRC1 inhibits MgcRacGAP activity through interaction with the basic region, and from the anaphase onward, perhaps PRC1 is unable to inhibit GAP activity through Aurora B phosphorylation of the basic region of MgcRacGAP. To clarify the role of the basic region of MgcRacGAP more, further analysis is necessary.

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References

1. Hyman, A. A., and Karsenti, E. (1996) Cell 84, 401–410
2. Glotzer, M. (2001) Annu. Rev. Cell Dev. Biol. 17, 351–386
3. O’Connell, C. B., Wheatley, S. P., Ahmed, S., and Wang, Y. L. (1999) J. Cell Biol. 144, 305–313
4. Kimura, K., Tseu, T., Takada, Y., Miki, T., and Narumiya, S. (2000) J. Biol. Chem. 275, 17233–17236
5. Madaule, P., Eda, M., Watanabe, N., Fujisawa, K., Matsuoka, T., Bito, H., Ishizaki, T., and Narumiya, S. (1998) Nature 394, 491–494
6. Matsumura, F., Totsukawa, G., Yamakita, Y., and Yamashiro, S. (2001) Cell Struct. Funct. 26, 639–644
7. Eda, M., Yonemura, S., Kato, T., Watanabe, N., Ishizaki, T., Madaule, P., and Narumiya, S. (2001) J. Cell Sci. 114, 3273–3284
8. Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kikuzuka, A., Saito, Y., Nakao, K., Jockusch, B. M., and Narumiya, S. (1997) EMBO J. 16, 3044–3056
9. Watanabe, N., Kato, T., Fujita, A., Ishizaki, T., and Narumiya, S. (1999) Nat. Cell Biol. 1, 136–143
10. Pelham, R. H., and Chang, F. (2002) Nature 419, 82–86
11. Bishop, A. L., and Hall, A. (2000) Biochem. J. 348, 241–255
12. Dutartre, H., Davoust, J., Gorce, J. P., and Chavrier, P. (1996) J. Cell Sci. 109, 367–377
13. Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A., and Hall, A. (2001) J. Biol. Chem. 276, 1677–1680
14. Wittmann, T., and Waterman-Storer, C. M. (2001) J. Cell Sci. 114, 3785–3803
15. Fujita, M., Watanabe, T., Nishihara, K., Nakagawa, M., Yamagushi, M., Kuroda, S., Matsuura, Y., Iwashita, A., Perez, F., and Kaibuchi, K. (2002) Cell 109, 873–885
16. Takai, Y., Sasaki, T., and Matsuzaki, M. (2001) Physiol. Rev. 81, 153–208
17. Tatsuno, T., Xie, X., Blumenthal, R., Okamoto, I., and Miki, T. (1999) J. Cell Biol. 147, 921–928
18. Hirose, K., Kawashima, T., Iwashita, I., Nosaka, T., and Kitamura, T. (2001) J. Biol. Chem. 276, 5821–5828
19. Kitamura, T., Kawashima, T., Minoshima, Y., Tonozuka, Y., Hirose, K., and Nosaka, T. (2001) Cell Struct. Funct. 26, 645–651
20. Toyoshima, K., Fujisawa, K., Tseu, T., and Glotzer, M. (2002) Dev. Cell 2, 877–885
21. Kawashima, T., Hirose, K., Satoh, T., Kaneko, A., Ikeda, Y., Kaziro, Y., Nosaka, T., and Kitamura, T. (2000) Blood 96, 2116–2124
22. Minoshima, Y., Kawashima, T., Hirose, K., Tonozuka, Y., Kawajiri, A., Bao, Y. C., Deng, X., Tatsuka, M., Narumiya, S., May, W. S., Nosaka, T., Semba, K., Inoue, T., Satoh, T., Inagaki, M., and Kitamura, T. (2000) Dev. Cell 4, 549–560
23. Jiang, W., Jimenez, G., Wells, N. J., Hope, T. J., Wahl, G. M., Hunter, T., and Fukagawa, R. (1998) Mol. Cell 2, 877–885
24. Molinari, C., Klemm, J. P., Jiang, W., Schoehn, G., Hunter, T., and Margolis, R. L. (2002) J. Cell Biol. 157, 1175–1186
25. Mitsuoka, M., Kaitina, S., and Glotzer, M. (2002) Dev. Cell 2, 41–54
26. Adams, R. R., Carmina, M., and Earnshaw, W. C. (2001) Trends Cell Biol. 11, 49–54
27. Terada, T. (2001) Cell Struct. Funct. 26, 653–657
28. Carnero, S. B., Urano, T., and Feg, L. A. (1990) Mol. Cell. Biol. 15, 4578–4584
29. Sugiyama, K., Sugiiro, K., Harata, T., Sugimoto, K., Shima, H., Honda, K., Furukawa, K., Yamashita, S., and Urano, T. (2002) Oncogene 21, 3103–3111
30. Eckley, D. M., Ainsstein, A. M., Mackay, A. M., Goldberg, I. G., and Earnshaw, W. C. (1997) J. Cell Biol. 136, 1169–1183
31. Mackay, A. M., Ainsstein, A. M., Eckley, D. M., and Earnshaw, W. C. (1998) J. Cell Biol. 140, 991–1002
32. Adams, R. R., Eckley, D. M., Vagnarelli, P., Wheatley, S. P., Gerloff, D. L., Mackay, A. M., Svingare, P. A., Kaufmann, S. H., and Earnshaw, W. C. (2001) Chronosoma 116, 65–74
33. Wheatley, S. P., Carvalho, A., Vagnarelli, P., and Earnshaw, W. C. (2001) Cell Biol. 11, 886–896
34. Honda, R., Kornber, R., and Nigg, E. A. (2003) Mol. Biol. Cell 14, 3235–3241
35. Jantsch-Plunger, V., Gonczy, P., Romano, A., Schnabel, R., Hamill, D., Schnabel, R., Hyman, A. A., and Glotzer, M. (2000) J. Cell Biol. 149, 1391–1404
36. Czetner, S., and Clermont, J. (2002) Genome Biology http://genomebiology.com/2002/2/6/reviews/0002.1
37. Belmont, L. D., and Mitchison, T. J. (1996) Cell 84, 623–631
38. Horwitz, S. B., Shen, H. J., He, L., Dittmar, P., Noell, R., Chen, J., and Schubart, U. K. (1997) J. Biol. Chem. 272, 8129–8132
39. Larsson, N., Marklund, U., Grudin, H. M., Brattsand, G., and Gullberg, M. (1997) Mol. Cell. Biol. 17, 5530–5539
40. Gavert, O., Ozon, S., Manceau, V., Lawler, S., Curmi, P., and Sobel, A. (1998) J. Cell Sci. 111, 3333–3346
41. Mistry, S. J., and Atweh, G. F. (2002) Mt. Sinai J. Med. 69, 299–304
42. Carmina, M., and Earnshaw, W. C. (2003) Nat. Rev. Mol. Cell Biol. 4, 842–854.
Human Mitotic Spindle-associated Protein PRC1 Inhibits MgcRacGAP Activity toward Cdc42 during the Metaphase
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