

The Rho-associated protein kinase p160ROCK is required for centrosome positioning

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The p160-Rho-associated coiled-coil-containing protein kinase (ROCK) is identified as a new centrosomal component. Using immunofluorescence with a variety of p160ROCK antibodies, immuno EM, and depletion with RNA interference, p160ROCK is principally bound to the mother centriole (MC) and an intercentriolar linker. Inhibition of p160ROCK provoked centrosome splitting in G1 with the MC, which is normally positioned at the cell center and

shows little motion during G1, displaying wide excursions around the cell periphery, similar to its migration toward the midbody during cytokinesis. p160ROCK inhibition late after anaphase in mitosis triggered MC migration to the midbody followed by completion of cell division. Thus, p160ROCK is required for centrosome positioning and centrosome-dependent exit from mitosis.

Introduction

Centrosomes, the major microtubule-organizing centers in mammalian cells, are complex organelles, comprising two centrioles associated by the pericentriolar matrix. Centrosomes are involved in both cell motility and cell division. In migrating cells, centrosome positioning is involved in the stabilization of pseudopodia (Ueda et al., 1997). Centrosomes are subject to important structural modifications during the cell cycle, including duplication, maturation, and separation (for reviews see Andersen, 1999; Tassin and Bornens, 1999; Hinchcliffe and Sluder, 2001).

Recent studies have revealed differential behavior of the two centrioles (Piel et al., 2000, 2001). In G1 cells, although the mother centriole (MC) stays close to the cell center, the daughter centriole (DC) shows wide excursions throughout the cell cytoplasm. The MC is responsible for the formation of a microtubule aster, and this is probably the basis for its positioning at the cell center (Holy et al., 1997). In contrast, the DC nucleates microtubules that are released and migrate toward the cell periphery (Piel et al., 2000). This combination of a centered, microtubule-anchoring MC

and of a mobile, microtubule-releasing DC could be important for directional cell motility. In addition, centrosome organization and motions are apparently crucial for the completion of cytokinesis. The separation of daughter cells at the end of mitosis is apparently controlled by a migration of the MC toward the midbody (Piel et al., 2001).

Some of the factors important for the control of centrosome organization and centrosome behavior have been identified. The ability of the MC to generate microtubule asters is attributable to specific features of its associated pericentriolar material (PCM),* which contains the microtubule-anchoring protein ninein (Mogensen et al., 2000; Piel et al., 2000). The relative position of the two centrioles and the centrosome movements within cells are dependent on both microtubule and microfilaments organization (Euteneuer and Schliwa, 1985; Schliwa et al., 1999). Centrosome cohesion is affected by the kinase Nek2 (Fry et al., 1998a,b; Mayor et al., 2000; Meraldi and Nigg, 2001). The identification of additional centrosome regulators may arise from an inventory of the centrosome components. To identify PCM components, we have previously developed monoclonal antibodies

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Key words: centrosome; structure; motion; p160ROCK; cytokinesis

*Abbreviations used in this paper: CD, cytochalasin D; DC, daughter centriole; GFP, green fluorescent protein; MC, mother centriole; nt, nucleotide; ROCK, Rho-associated coiled-coil-containing protein kinase; PCM, pericentriolar material.

directed against isolated centrosomes. One of these antibodies, mAb 6C6, reacted with the centrosome or microtubule-organizing centers in a wide variety of animal and plant cells (Chevrier et al., 1992). Here, using this antibody we have identified the Rho-dependent protein kinase p160ROCK as a PCM component. p160ROCK is important for actin organization and coordinated microtubule regulations (Ishizaki et al., 1996, 1997; Matsui et al., 1996; Narumiya et al., 1997; Hirose et al., 1998; Amano et al., 2000; Kosako et al., 2000). We report that p160ROCK is also required for centriole positioning and is essential for MC movement toward the midpiece in late telophase, hence for signaling the end of cytokinesis.

Results

Centrosomal localization of p160 ROCK

In a screen of an MDBK cDNA expression library with mAb 6C6, one positive clone (clone N) coded for a 438 amino acid polypeptide showing 98% sequence homology with a peptide sequence belonging to the kinase, p160ROCK (Fig. 1 A). This data raised the possibility that p160ROCK was present on centrosomes. To test this possibility, protein N was expressed in bacteria and used to generate a polyclonal antibody (antibody N). Epitopes recognized by the antibody were then mapped using peptide array analysis (SPOT analysis) (Fig. 1 B). Nine peptide clusters reacted with the antibody N on SPOT membranes (peptides N1–N9). Five peptide-specific antibodies were further derived from the antibody N using affinity chromatography with peptides N2, N3, N5, N6, and N7, respectively (Fig. 1 C). On Western blots of total protein extracts from MDBK cells, these antibodies reacted with a single 160-kD band corresponding to p160ROCK (Fig. 2 A). The same antibodies reacted with a 160-kD band on immunoblots of centrosomal fractions and also reacted with an additional protein of slightly smaller apparent molecular weight (Fig. 2 A). This latter protein may be a proteolytic product of p160ROCK generated during centrosome preparation or a specific centrosomal variant of p160ROCK whose structure remains to be determined. The antibodies also brightly stained centrosomes in different cell lines including MDBK and HeLa cells. The staining was concentrated on the MC and on an apparent linker structure between the centrioles (Fig. 2 B). Similar centrosome staining was observed with other p160ROCK antibodies, including commercial antibodies, ROCK-1 (C-19) antibody, and an affinity purified antibody raised against peptide N6-stained centrosomes (unpublished data). Immuno EM examination of centrosomes isolated from human KE37 lymphoblastic cells also showed intense staining of the intercentriolar linker (Fig. 2 C). The difference in labeling between the mother and DC was not clear on immuno EM images may be due to some degree of PCM dispersion during centrosome purification and absence of signal summation in centrosome sections.

We used RNA interference to test directly whether the centrosomal staining observed with p160ROCK antibodies corresponded to p160ROCK. HeLa cells were transfected with four different silencing RNA (siRNA) oligoduplexes corresponding to different coding regions of the p160ROCK

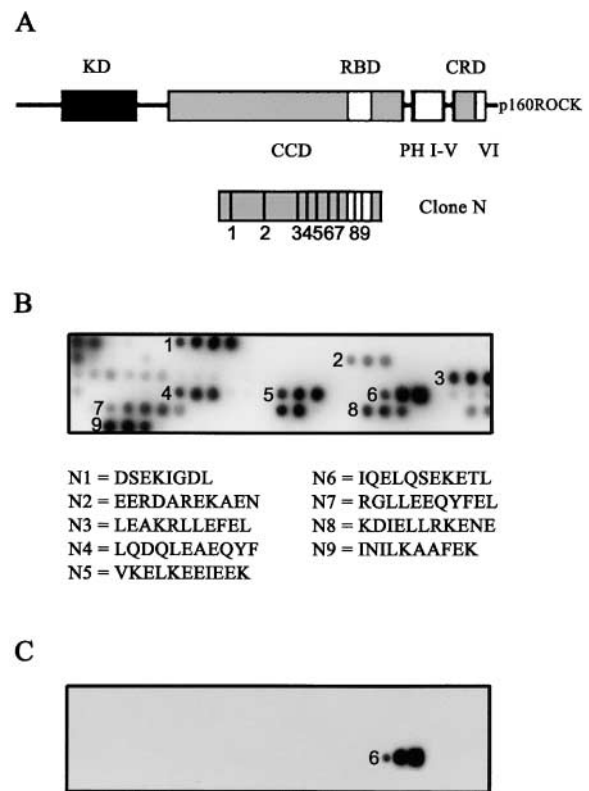


Figure 1. Structure of clone N. (A) Schematic representation of p160ROCK and clone N. p160ROCK (sequence data available from GenBank/EMBL/DDBJ under accession no. U43195) is a 160-kD protein serine/threonine kinase containing a kinase domain (KD), a long amphipathic α -helix capable of forming a coiled-coil structure (CCD) containing a Rho binding domain (RBD), the pleckstrin homology domain (PHI-V, VI) split by a cysteine-rich zinc finger (CRD); clone N (sequence data available from GenBank/EMBL/DDBJ under accession no. AY025529) coded for a protein fragment (protein N) corresponding to amino acids 594–1028. The positions of the nine epitopes recognized by polyclonal antibody directed against protein N (see B) are indicated. (B) Peptide SPOT analysis of the epitopes recognized by protein N polyclonal antibody showing nine positive peptide clusters (1–9). The sequences of the nine corresponding epitope peptides are shown below (N1 to N9). (C) Peptide SPOT analysis of the affinity purified N6 antibody. The affinity purified antibody reacted exclusively with peptide N6.

mRNA. Immunoblot analysis of total cell extracts showed p160ROCK depletion in cells exposed to siRNA (Fig. 2 D). In the presence of each one of the siRNA oligoduplexes, a substantial proportion of cells showed nuclear condensation and major disturbances of the microtubule cytoskeleton. These cells were not further analyzed. Among cells with apparently normal nuclei and microtubule content, cells with a conspicuously abnormal morphology, typical of p160ROCK inhibition (Hirose et al., 1998; Tominaga et al., 1998) were observed. Such cells have an irregular or elongated shape with characteristic cell extensions (Fig. 2 E; see Fig. 4, A and B). For each one of the four siRNA duplexes, a total of <100 cells showing abnormal morphology were examined for centrosome staining with p160ROCK antibody. This centrosome staining was uniformly absent. Cells transfected with mutated siRNA duplexes had normal morphology and always showed centrosome staining with p160ROCK antibody as nontransfected control cells (unpublished data).

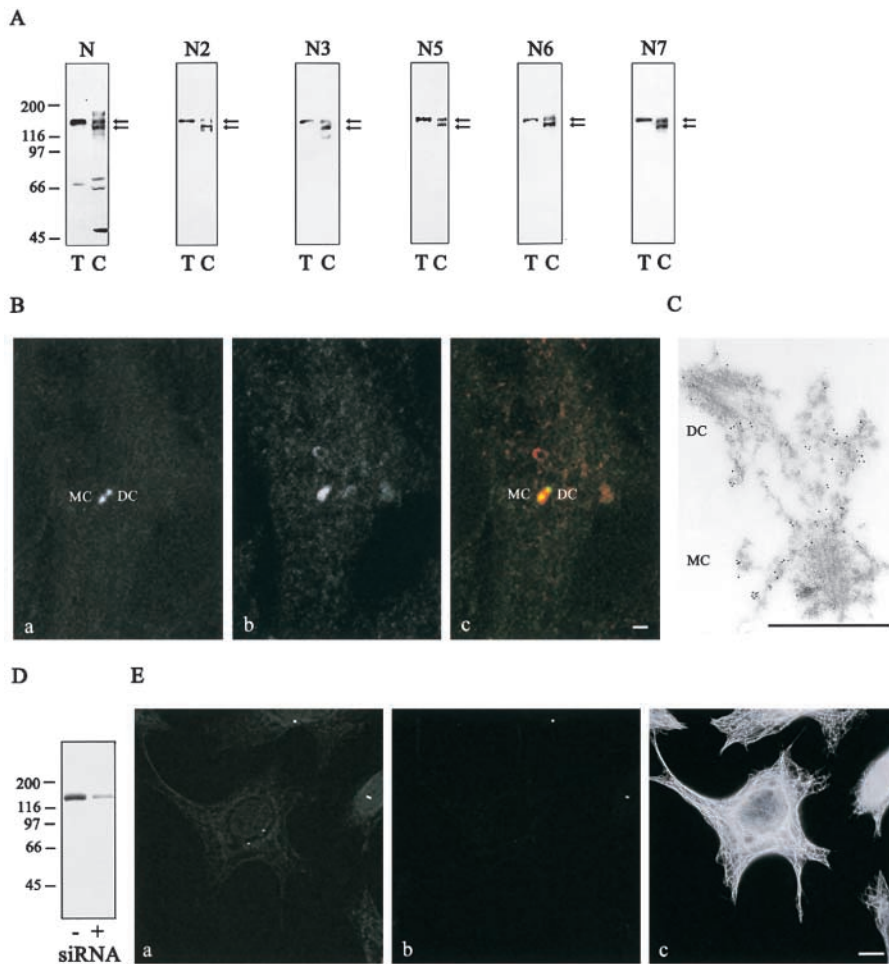


Figure 2. Centrosomal localization of p160ROCK. (A) Immunoblot analysis of total protein extracts from MDBK cells (T) and purified calf thymus centrosomes (C) using antibodies N, N2, N3, N5, N6, and N7. In total cell extracts, the peptide-purified antibodies reacted with a single 160-kD protein corresponding to p160ROCK. In centrosomal fractions, the same antibodies also stained a slightly lower molecular weight protein that may be a proteolytic product of this protein. (B) Immunostaining of HeLa GFP-centrin cells (a) with N6 antibody (b). The MC and DC were identified based on the centrin signal by conventional fluorescence microscopy examination of the cells. On visual examination, the MC shows distinctly stronger centrin signal than the DC (Piel et al., 2000). This difference is still visible although often less striking in confocal images. Bar, 1 μm . (C) Immuno EM localization of p160ROCK with polyclonal antibody N6 in isolated KE 37 centrosomes showing staining of the PCM, including the intercentriolar linker. Controls run with secondary antibody alone showed a complete absence of staining (unpublished data). Bar, 1 μm . (D) Silencing of p160ROCK: immunoblot analysis of p160ROCK in total cell extracts from either control HeLa cells or from HeLa cells transfected with siRNA n°3 (72 h after transfection), using p160ROCK antibody N6 as a primary antibody. Equal amounts of total cell protein from

control and transfected cells were loaded as estimated from Coomassie blue gels (unpublished data). (E) Centrosome staining in HeLa GFP-centrin cells transfected with siRNA: cells were transfected with siRNA n°3. (a) Centriole visualization with GFP-centrin; (b) centriole staining with p160ROCK N6 antibody; (c) microtubule staining with anti- β -tubulin antibody. Changes in cell shape were used as a criteria for p160ROCK depletion. The figure is centered on a cell with abnormal shape, showing absence of centrosome staining with p160ROCK antibodies. The cell was surrounded by cells of normal shape, showing p160ROCK centrosome staining. Bar, 5 μm .

These results yield compelling evidence for a centrosomal localization of p160ROCK.

Effect of p160ROCK inhibition on centriole splitting

The centrosomal localization of p160ROCK raised the possibility that p160ROCK activity could affect centrosome structure or activity. We used several independent methods of p160ROCK inhibition to test this possibility.

We first examined the effect of the p160ROCK inhibitor Y-27632 (Uehata et al., 1997) in HeLa cells stably expressing green fluorescent protein (GFP) centrin stained with antibody N6 (Fig. 3). In control cells, centrosome staining was observed at all phases of the cell cycle. In late G2 cells, both centrosomes were stained, and the specific labeling of the linker structure evident in G1 was not visible (Fig. 3, d–f). Centriole staining with antibody N6 persisted through mitosis and cytokinesis. In the presence of Y-27632, the intercentriolar distance in G1 cells was generally conspicuously increased compared with controls (Fig. 3, a–c and a'–c'). When centrioles were split, the MC staining persisted, but no distinct staining of a linker structure was detectable (Fig.

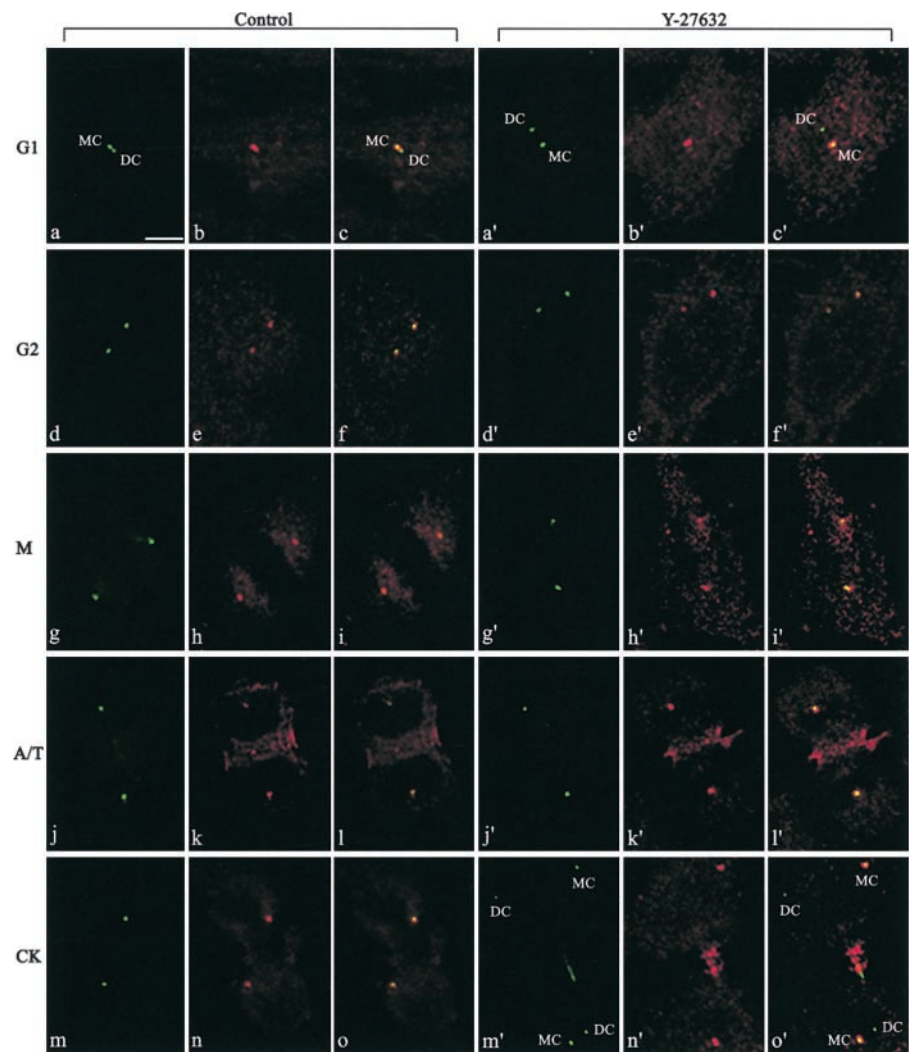
3, a'–c'). Y-27632 had no apparent influence on centrosome staining in G2, metaphase, or anaphase cells. In telophase cells, treatment with Y-27632 induced a concentration of p160ROCK labeling in the cell midzone (Fig. 3, j'–o').

The intercentriolar distance increased in cells treated with 10 μM Y-27632 (Fig. 4 A, f and h), but the drug effect was maximal with 100 μM Y-27632 (Fig. 4 A, j and l). Treated cells tended to elongate microtubules to form parallel bundles (Fig. 4 A, e and i), whereas actin stress fibers disappeared (Fig. 4 A, g and k). After drug withdrawal, microfilament and microtubule networks and the cell shape apparently went back to normal within 2–4 h (Fig. 4 A, m and o). In contrast, the intercentriolar distance remained elevated (Fig. 4 A, n and p).

When cells were transfected with the p160ROCK mutant KDIA, which acts as a dominant negative (Ishizaki et al., 1997), similar morphology changes and centrosome splitting (Fig. 4 B) were observed as in Y-27632-treated cells. Similar changes were also observed with siRNAs (Fig. 2 E).

In quantitative study of p160ROCK inhibition, the average intercentriolar distance increased threefold in the presence of 100 μM Y-27632 (Fig. 5 A) with a half-maxi-

Figure 3. Cell cycle changes in the distribution of p160ROCK. Immunofluorescence of HeLa GFP-centrin cells with N6 antibody. Centrioles are visible as green dots (a, a', d, d', g, g', j, j', m, and m'). The red staining corresponds to N6 antibody staining (b, b', e, e', h, h', k, k', n, and n'). Merged images: c, c, f, f', i, i', l, l', o, and o'. Cells are shown in G1 phase (a–c and a'–c'), G2 phase (d–f and d'–f'), metaphase (M; g–i and g'–i'), late anaphase through early telophase (A/T; j–l and j'–l'), and cytokinesis (CK; m–o and m'–o') in the absence (a–o) or presence (a'–o') of the p160ROCK inhibitor Y-27632. The MC and DC were identified as in Fig. 2. Bar, 5 μ m.



mal effect within 45 min after cell exposure to the drug. After drug withdrawal, no significant changes in the average intercentriolar distance were observed within 4 h (Fig. 5 B). Cell shape changes were quantified, using as an index the ratio of the cell perimeter to the cell surface (Fig. 5 C). When cells were treated for 1.5 h with 100 μ M Y-27632, the perimeter to surface ratio increased significantly in the presence of the drug but went back to control values within 2 h after drug withdrawal, whereas centriole separation persisted (Fig. 5, B and C).

Increases of the perimeter to surface ratio and of the intercentriolar distance were also observed in cells transfected with the KDIA construct or with siRNA (Fig. 5, D and E). Thus, similar centrosome splitting was observed using three independent methods for p160ROCK inhibition.

Effect of p160ROCK inhibition on centrosome positioning

We used videomicroscopy to investigate the effect of p160ROCK inhibition on centriole positioning. Centrioles display little motion during the S and G2 phases of the cell cycle. In contrast, centrioles can show important motion in G1 when the DC transiently shows excursion through the cell cytoplasm in migrating cells, and in late telophase, when

the MC leaves its central position and migrates toward the midbody (Piel et al., 2001). Cell exposure to 100 μ M Y-27632 did not change centriole behavior in S or G2 cells (unpublished data). In late anaphase cells exposed to the drug, the MC movement toward the midbody remained of normal amplitude and duration (Fig. 6). In contrast, after abscission and exit from mitosis major changes affected the MC behavior, which instead of being almost immobile and positioned near to the cell center as in control cells displayed persistent motion (Fig. 6, A and B), often being located near the cell periphery. Although in control cells both centrioles remained in close vicinity, except for short periods of time (Fig. 6 A, 03:24 and 03:28, and B), in treated cells the two centrioles were often widely separated (Fig. 6, A and B). After drug withdrawal, the MC behavior was back to normal within 2 h, and the organelle slowly relocated to the cell center. However, the two centrioles usually failed to cluster, and the intercentriolar distance remained elevated (Fig. 6 B).

In G1 cells treated with Y-27632 after cytokinesis, the MC left its normal central position within a few minutes after addition of the drug, moving away from the DC and undergoing erratic motion. The MC frequently moved back and forth along the plasma membrane (Fig. 7 B). In some cases, it circled the nucleus (Fig. 7 B).

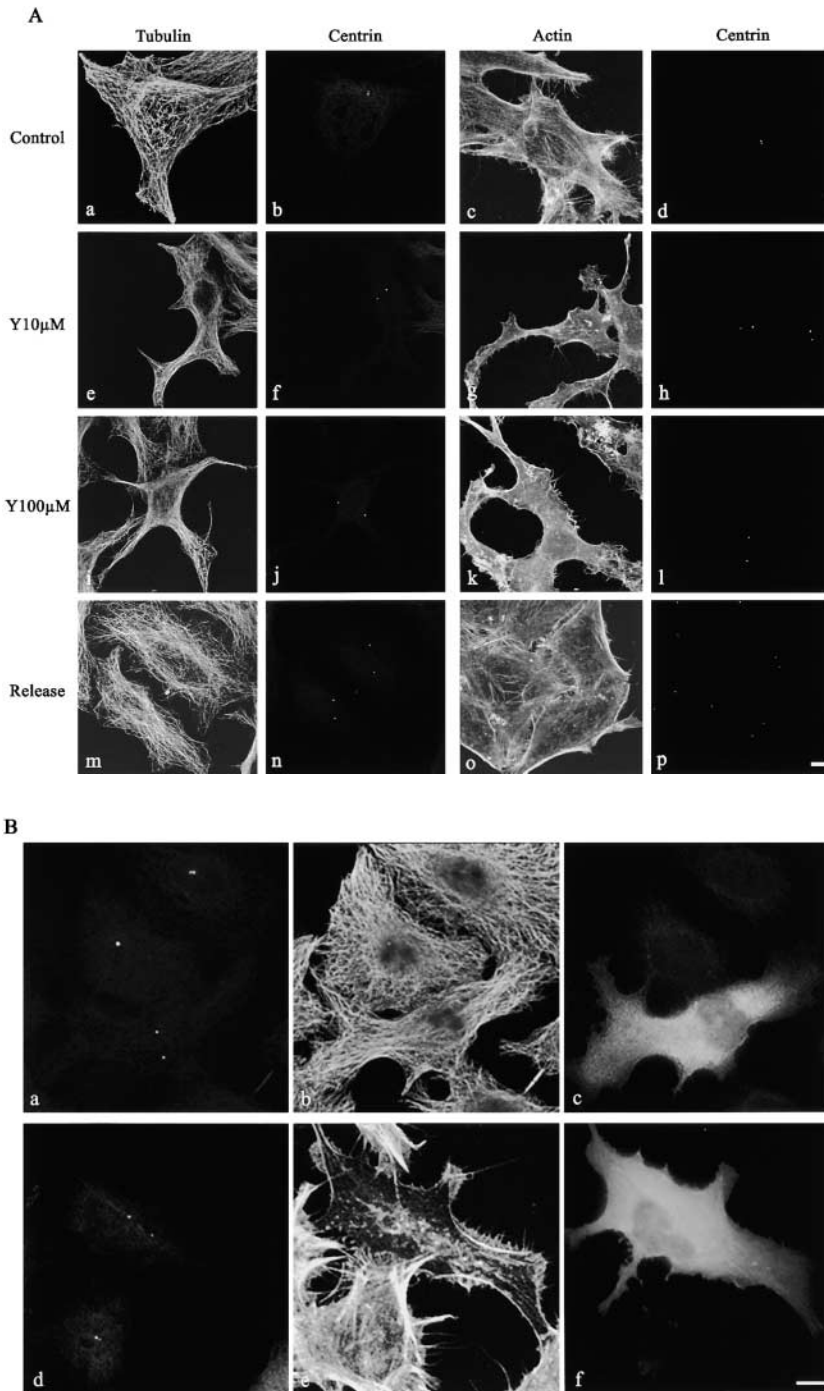


Figure 4. Centrosome splitting by p160ROCK inhibition. (A) Immunostaining of HeLa GFP-centrin cells with monoclonal anti- β -tubulin antibody or phalloidin-rhodamine. Untreated cells (a–d); cells treated for 1.5 h with 10 μ M Y-27362 (e–h); cells treated with 100 μ M Y-27362 for 1.5 h (i–l); cells treated with 100 μ M Y-27362 for 1.5 h (m–p) examined 4 h after withdrawal of the drug. Exposure to the drug caused cell shape changes, stress fiber disruption, and centrosome splitting. Centrosome splitting persisted after drug withdrawal. Bar, 5 μ m. (B) Immunostaining of HeLa GFP-centrin cells transfected with cMyc-tagged p160ROCK dominant negative KDIA construct. Cells were examined 24 h after transfection. GFP-centrin (a and d); tubulin staining (b); phalloidin-rhodamine staining (e); c-Myc staining (c and f). c-Myc-positive cells showed abnormal shape, stress fiber disruption, and centrosome splitting as Y-27632-treated cells. Bar, 5 μ m.

In the presence of nocodazole, the effect of Y-27632 on the MC movement was inhibited (Fig. 8). In contrast, a 30-min treatment with cytochalasin D (CD) preceding Y-27632 addition did not modify the MC behavior. Thus, the MC movements induced by Y-27632 were microtubule dependent but actin independent as reported previously for the telophase MC movement (Piel et al., 2001). Cytochalasin treatment suppressed the DC motion (Fig. 8) as previously observed (Piel et al., 2000).

Centriole movements were also examined in cells transfected with KDIA mutant. In transfected G1 cells, MCs showed the same erratic motions around the cells as in cells treated with Y-27632 (unpublished data).

p160ROCK inhibition triggers MC migration toward the midpiece and subsequent completion of cytokinesis in postanaphase cells

After our observations of centriole separation and MC delocalization after p160ROCK inhibition, we wondered whether p160ROCK inhibition could trigger MC migration in the midbody in postanaphase cells. To test this possibility, postanaphase cells isolated by mitotic shake off were treated with Y-27632 1 h after replating, and the percentage of pairs of daughter cells with a centriole in the midbody region bridge was monitored over time (Fig. 9 A). The percentage of cells linked by a cytoplasmic bridge was determined at the same time points (Fig. 9 B). Centriole

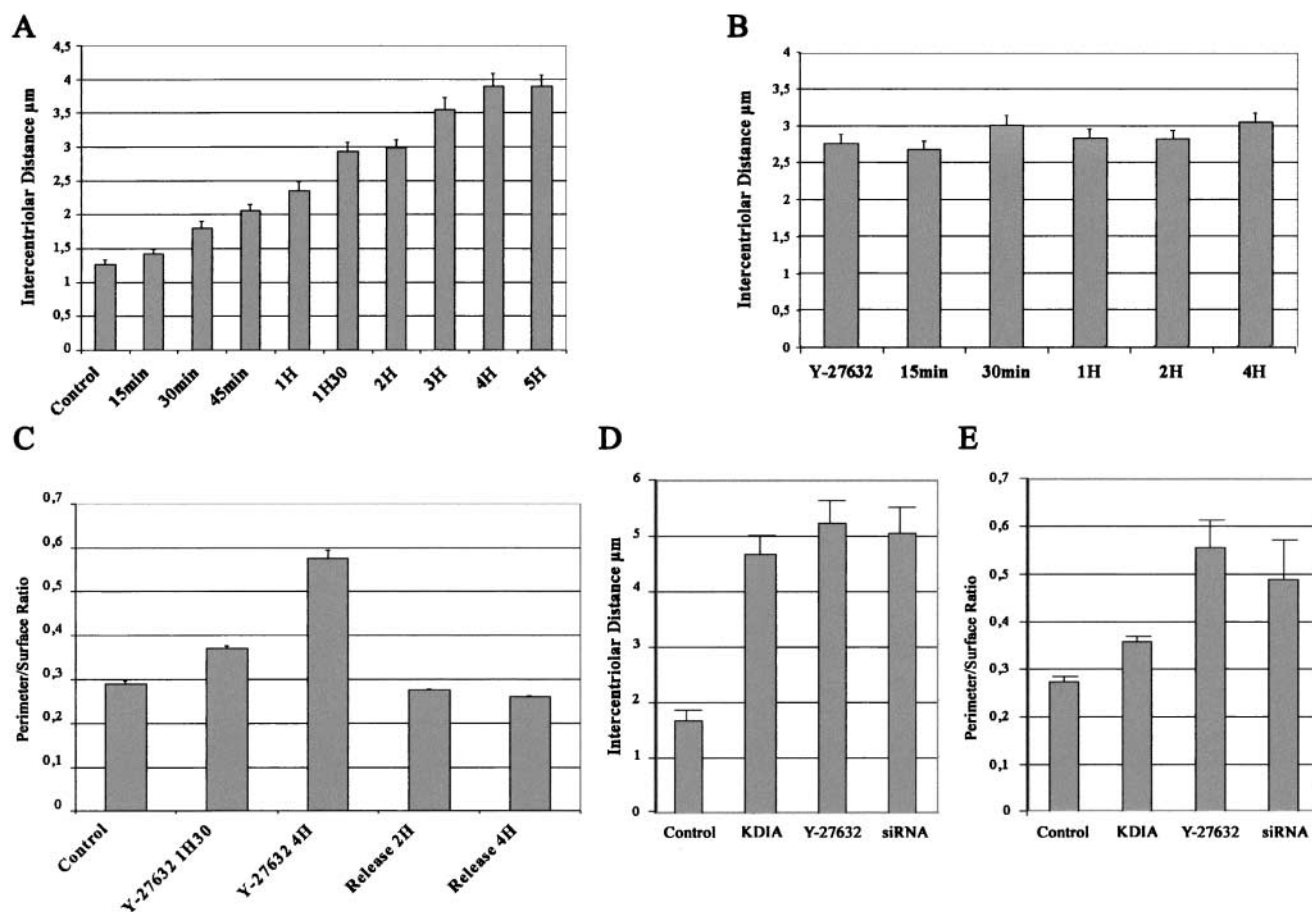


Figure 5. Quantitative analysis of intercentriolar distance and cell shape after p160ROCK inhibition. (A) Average intercentriolar distances in cells treated with 100 μ M Y-27632 for the indicated amounts of time. In each condition, the average intercentriolar distance was measured on a minimum number of 200 cells. SEMs are indicated. (B) Average intercentriolar distances in cells treated with 100 μ M Y-27632 for 1.5 h and then left to recover for the indicated amounts of time after drug withdrawal. Cell numbers and SEMs are as in A. (C) Perimeter to surface ratios in cells treated with 100 μ M Y-27632. Cells were treated with Y-27632 for the indicated amounts of time and then fixed and processed for analysis. In release experiments, cells were treated with 100 μ M Y-27632 for 90 min, then the drug was withdrawn, and at the indicated times after release from the drug, cells were fixed for subsequent analysis. In each condition, a minimum of 50 cells was examined. The perimeter to surface ratios showed little dispersion, and in some conditions SEMs were too small to be visible on the figure. (D) Average intercentriolar distances after p160ROCK inhibition with 100 μ M Y-27632, KDIA mutant, or siRNA as indicated. Cells were treated with Y-27632 for 4 h before analysis. KDIA-transfected cells were analyzed 24 h after transfection. For siRNA-treated cells, the analysis (48 h after transfection) concerns cells with extinct p160ROCK signal, normal nuclei, and assembled microtubule arrays. In each condition, the average intercentriolar distance was measured on a minimum number of 100 cells. SEMs are indicated. (E) Perimeter to surface ratios after p160ROCK inhibition. Conditions were as in D.

migration in the midbody occurred prematurely in treated cells compared with controls with a frequency maximum at 30 min after exposure to the drug compared with 1.5–2 h in controls (Fig. 9 A). This maximum was up to 60% in drug-treated cell pairs compared with <15% in control cell pairs. Thus, there was a striking synchronization effect of drug treatment on MC localization in the midbody. Additionally, the proportion of cells linked by a cytoplasmic bridge dropped prematurely in drug-treated cells compared with control cells and then continued to diminish more rapidly over time (Fig. 9 B), indicating that the drug-induced MC migration to the midbody was followed by abscission.

Discussion

Centrosomal localization of p160ROCK

Our data show association of p160ROCK with the PCM

surrounding the MC and with an intercentriolar linker structure in G1 cells. Apparently, the DC recruits p160ROCK during duplication. Several proteins show transient or permanent-specific association with the MC including ninein (Mogensen et al., 2000), cenexin, or ODF2 (Lange and Gull, 1995; Nakagawa et al., 2001) and epsilon tubulin (Chang and Stearns, 2000). Our data identify p160ROCK as an additional marker of the MC.

On EM images of isolated centrosomes, the MCs and DCs are bridged by a distinct linker structure (Bornens et al., 1987). However, markers of this structure in intact cells have been lacking, and this has led to doubts concerning the existence of an intercentriolar linkage in vivo (Jean et al., 1999). Our data provide strong evidence for a localization of p160ROCK on the linker both in vitro and in vivo. Therefore, the enzyme emerges as a new marker of the linker in cells.

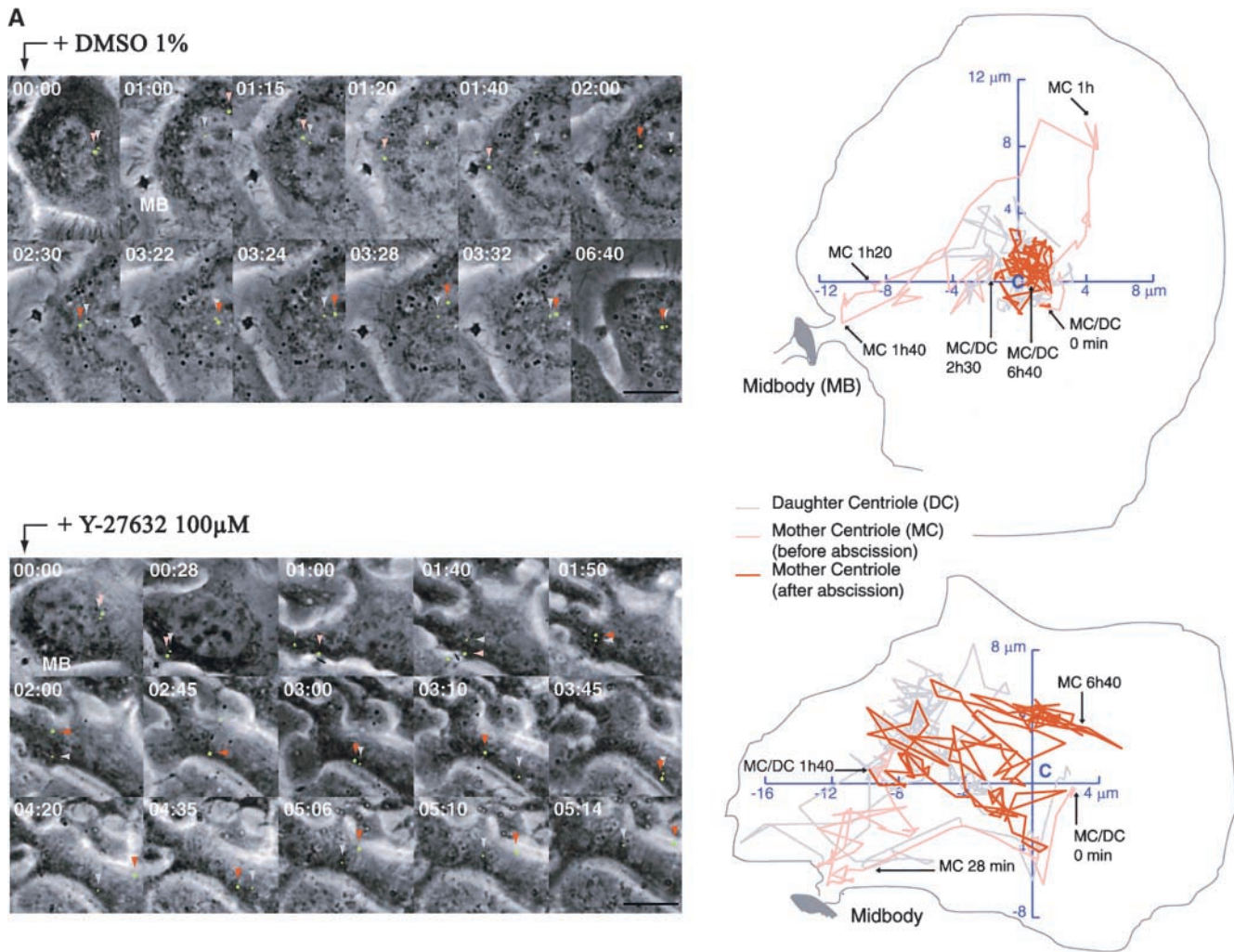


Figure 6 (continues on next page)

Effects of p160ROCK inhibition on centrosome structure and behavior

We find that p160ROCK inhibition triggers a permanent centriole splitting in G1, persisting for hours after release from inhibition when cells had recovered apparently normal cytoskeleton, shape, and centriole motion. This is a strong indication that p160ROCK inhibition causes changes in centrosome structure, namely in the intercentriolar linker, which seems to be an important determinant of the intercentriolar distance in G0-G1 cells (Komesli et al., 1989). The apparent irreversibility of the centrosome splitting and the disappearance of the p160ROCK staining of the intercentriolar area observed in G1 cell exposed to Y-27632 may correspond to a complete linker disruption. However, the loss of linker staining may be due to linker decondensation with resulting dilution of the signal. We have observed that even when centrioles are far apart in cells they seem to remain connected by a loose structure containing the matrix protein AKAP 450 and centrin (unpublished data).

We also find that p160ROCK inhibition induces wide MC excursions and relocation near the plasma membrane. This effect is actin independent but microtubule dependent and could involve a modification of microtubule dynamics

through the action of cytoplasmic p160ROCK on microtubule dynamics or organization (unpublished data). Nevertheless, the centrosomal localization of a fraction or an isoform of p160ROCK suggests an action on the centrosome itself. p160ROCK inhibition has no evident effect on microtubule nucleation (unpublished data) but may affect microtubule anchoring or the anchoring of the MC in the surrounding cytoplasm.

The MC movements induced in G1 cells by p160ROCK inhibition share close similarity with the normal telophase movement of this centriole. Both movements involve wide excursions and relocation of the MC at the cell periphery and show similar differential sensitivity to nocodazole and CD. Additionally, previous work has shown down-regulation of Rho at the end of telophase (Kimura et al., 2000). We now show that p160ROCK inhibition in postanaphase cells is sufficient to trigger MC migration to the midpiece and subsequent abscission. Together, these data strongly indicate a central involvement of p160ROCK in triggering centrosome-dependent exit of mitosis in cycling cells.

In this study, Y-27632 and the p160ROCK dominant negative KDIA mutant had similar effects both inducing centrosome splitting and wide MC motility. This similarity in effects suggests a model in which centrosomal substrates

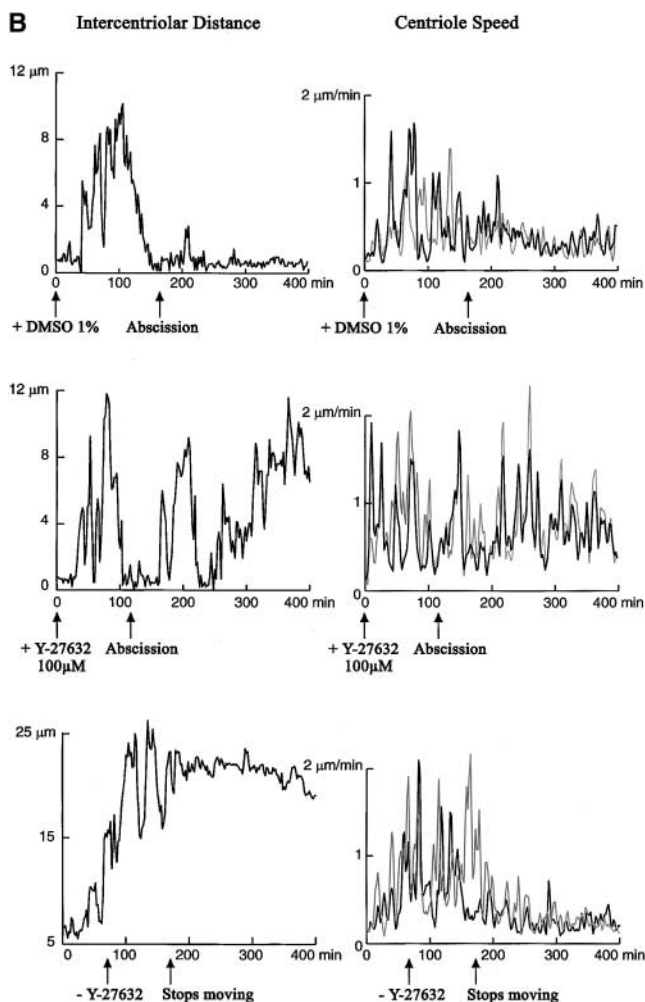


Figure 6. Videomicroscopy analysis of Y-27632 effect on centriole motility. Phase-contrast and fluorescence images of postmitotic and G1 HeLa cells stably expressing GFP-centrin were recorded every 2 min. Cells were placed in a medium containing either DMSO (control cells) or Y-27632 as indicated. (A) The images on the left are extracted from movies. The corresponding time is indicated on the top left corner of each frame (h:min). An overlay of GFP signal (green) on the corresponding phase-contrast image is shown. The centrioles were spotted by hand with green dots to allow visualization at low magnification. The MC is the bigger one, indicated by a pink arrowhead before midbody rupture and a red arrowhead after midbody rupture. The DC is indicated by a gray arrowhead. Centriole trajectories are drawn on the right with the same color code. The cell margin at the beginning of the movie is indicated by a thin gray line. Thanks to the cytoplasmic fluorescence of the soluble GFP-centrin pool, the cell shape could be tracked automatically, and then the centroid of the cell was calculated for each plane. Centriole position is shown relatively to that centroid, which is thus the point 0 of the coordinates (indicated by C). Y-27632 was added at time 00:00. Bar, 10 μm . (B) The intercentriolar distance and the centriolar speed are plotted over time (gray curve, DC speed). The four top graphs correspond to the cells shown in A. The two bottom graphs correspond to a release experiment: Y-27632 was added 1 h before time 0, and then the cells were filmed during 1 h before the medium was replaced by Y-27632-free medium (three washings). In the case shown, the intercentriolar distance is elevated because the cell was elongated and the centrioles were on both sides of the cells when the drug was removed, resulting in an arrest of centriolar motion. The centrioles never clustered again after drug removal.

of p160ROCK are also binding partners of the enzyme. Y-27632 is known to inhibit p160ROCK catalytic activity by competing with ATP (Narumiya et al., 2000). In the presence of the drug, the unphosphorylated p160ROCK substrate partners may remain bound on the enzyme and never assemble into PCM structures. The same partners may be trapped by the cytoplasmic KDIA mutant and, in the presence of this mutant, never reach the centrosome.

SiRNA duplexes had additional deleterious effect on cells compared with Y-27632 or KDIA mutants, ultimately inducing cell death. This probably results from the complete elimination of all p160ROCK domains by RNA interference, whereas the two other methods leave extensive parts of the protein intact.

In conclusion, our data provide strong evidence that p160ROCK activity is important for both centrosome organization and positioning of the centrioles and that it plays a central role in centrosome-dependent cell exit of mitosis.

Materials and methods

Library screening and cDNA cloning and sequencing

A MDBK cDNA expression library (CLONTECH Laboratories, Inc.) was screened with a previously characterized monoclonal antibody mAb 6C6 (Chevrier et al., 1992) using standard procedures.

Expression and purification of recombinant proteins

The clone N cDNA was subcloned into pGEX-4T3 vector (Amersham Pharmacia Biotech). *Escherichia coli* BL21 cells were transformed with the pGex construct. Fusion protein induction and purification were done using standard methods.

Antibody production and purification

Immunization of rabbit was performed using 500 μg of purified N protein emulsified in complete Freund's adjuvant for the primary injection. Subsequent booster immunizations were performed on days 14, 28, 42, 56, and 70 using 500 μg of purified N protein emulsified in Freund's incomplete adjuvant. A test bleed demonstrated anticentrosome antibodies; pre-immune serum was also collected and used for some control studies.

Serum was affinity purified against the purified N protein coupled to sepharose 4B. The bound antibodies were eluted using 10 vol of 0.1 M glycine, pH 2.5. The eluted antibodies were neutralized by the addition of 1 vol 1 M Tris HCl, pH 8.0, and the antibodies were dialysed against PBS and concentrated for long term storage.

For anti-N peptide antibodies, serum was affinity purified against each corresponding peptide coupled to sepharose 4B, and they were eluted as described previously.

SPOT analysis

SPOT synthesis corresponding to N protein was performed according to Frank (1992) with an Abimed ASP 222 automated SPOT robot. Peptide sheet was permeabilized in ethanol bath, washed three times (10 min each) with PBS 0.1% Tween 20 (PBST), and incubated 1 h at room temperature with purified polyclonal antibody anti-N protein (1:5,000) or anti-peptides N(1-9) (1:5,000) in PBST. After three washes (10 min each) in PBST, the membrane was incubated with anti-rabbit antibody labeled with HRP (1:5,000), washed three times as above, and developed using the chemiluminescent ECL kit (Amersham Pharmacia Biotech).

RNA interference

To design target-specific siRNA duplexes, we selected four sequences of the type AA(N19)dTdT from the ORFs of the p160ROCK mRNA (U43195) in order to obtain a 21-nucleotide (nt) sense and 21-nt antisense strand with symmetric 2-nt overhangs of identical sequence as described by Harborth et al. (2001). We used 2' deoxythymidines instead of uridine residues in the 3' overhangs to enhance nuclease resistance. The selected sequences were submitted to a BLAST search against the human genome sequence to ensure that only p160ROCK gene of the human genome was targeted. 21-nt RNAs were purchased from Dharmacon in deprotected and desalted form. The siRNA sequence targeting p160ROCK were from position relative to the start

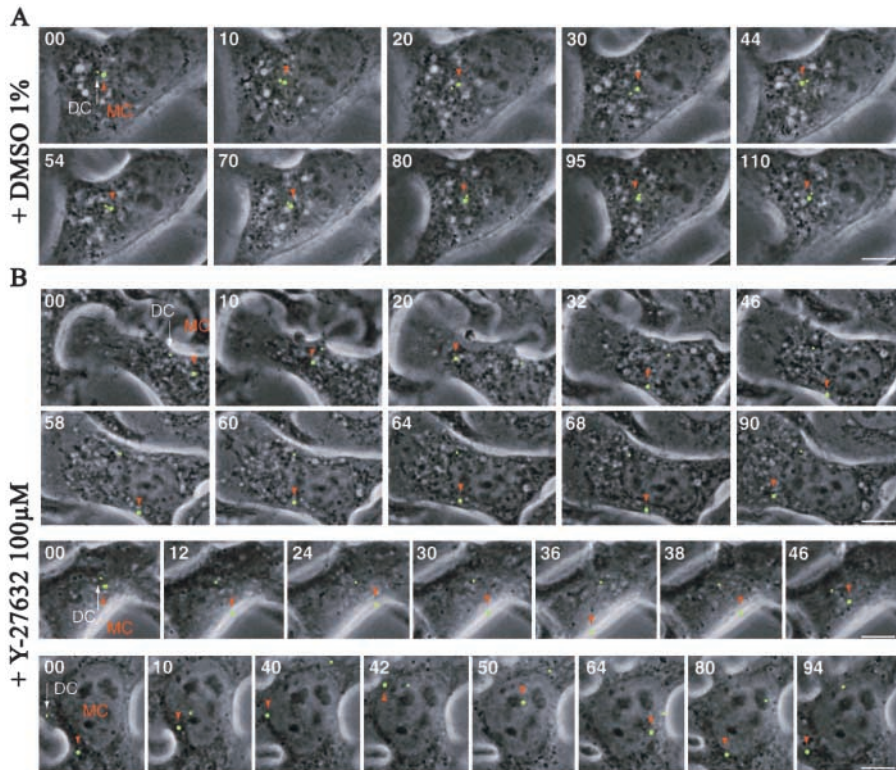


Figure 7. Centriole behavior in G1 HeLa cells. Centrioles are shown as in Fig. 6 A. G1 HeLa cells recorded as in Fig. 6. Time is in minutes. (A) Control cell; the MC stays near the cell center. (B) Three examples of characteristic behaviors of the MC in cells treated with 100 μ M of Y-27632: either running along the membrane (first two rows) or turning around the nucleus (last row). Bar, 10 μ m.

codon: n^o1 = 566–584, n^o2 = 639–657, n^o3 = 1958–1976, and n^o4 = 2780–2798. As unspecific siRNA control, we used siRNA n^o3 mutated on three nucleotides. For annealing, 20 μ M single-stranded 21-nt RNAs in annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37°C.

Cell culture and transfection

MDBK cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated bovine FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C with 6.5% CO₂.

HeLa cells stably expressing GFP-centrin were maintained using previously reported procedure (Piel et al., 2000).

To inhibit p160ROCK, cells were treated with 10 μ M or 100 μ M Y-27632 (provided by Yoshitomi Pharmaceutical Industries) for 15 min to 5 h at 37°C.

The day before transfection, cells were trypsinized, diluted with fresh medium, and transferred to 24-well plates (10⁴ cells/well). For transient transfection of siRNA, Oligofectamine (Invitrogen) was used. 12 μ l OPTIMEM medium (Invitrogen) and 3 μ l Oligofectamine per well was preincubated for 10 min at room temperature. In parallel, 50 μ l OPTIMEM medium was mixed with 3 μ l siRNA (60 pmole). The two mixtures were combined and incubated for 20 min at room temperature. After addition of

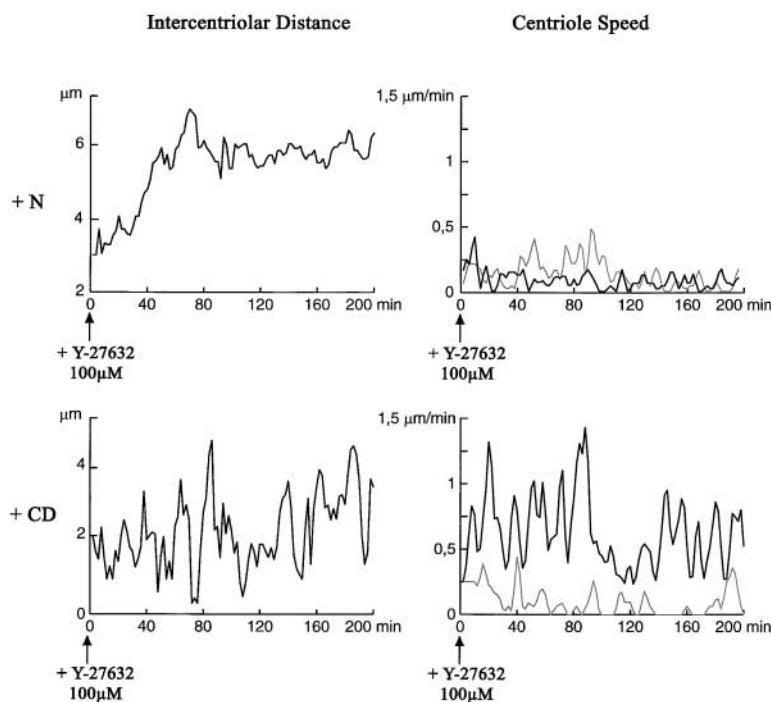


Figure 8. Videomicroscopy analysis of nocodazole and cytochalasin effect on centrosomes in cells treated with Y-27632. The intercentriolar distance and the centriolar speed are plotted over time (gray curve, DC speed). (Top graphs) G1 cells were treated with 5 μ M nocodazole (N) and cold for 30 min to depolymerize microtubules and then rewarmed at 37°C and filmed in a medium containing nocodazole and 100 μ M Y-27632. (Bottom graphs) Cells were treated with 1 mg/ml CD for 20 min and then filmed in the presence of CD and Y-27632.

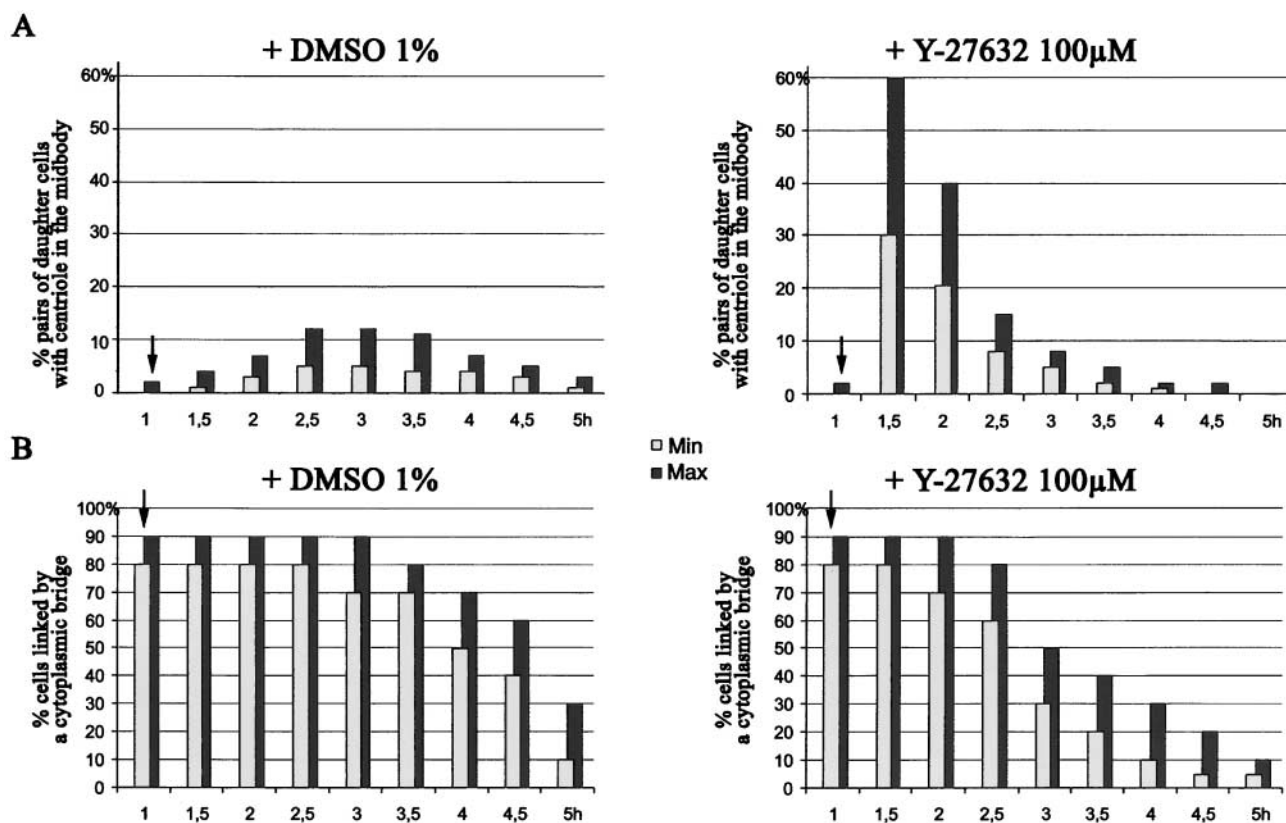


Figure 9. **Synchronization effect of p160 ROCK inhibition on MC movements and abscission.** Cells were treated either with 1% DMSO or 100 μ M Y-27632 1 h after replating from a mitotic shake off (arrows). Coverslips were then fixed at indicated time points, and the percentages of both daughter cell pairs with centriole in the midbody (A) and cells linked by a cytoplasmic bridge (B) were determined. At least 300 cells were counted for each percentage determination. Four independent experiments were done. The maximum (max) and the minimum (min) values observed are indicated. Percentages do not add up to 100% because centriole localization in the midbody can escape detection and because of the statistical error attached to each percentage determination.

32 μ l of OPTIMEM medium, the mixture was added to cells. Cells were usually assayed 48–72 h after transfection. For transient transfection with KDIA, a cMyc-tagged dominant negative mutant of p160ROCK (Ishizaki et al., 1997), Lipofectamine Plus reagent (Invitrogen) was used. Cells were transfected with 0.5 μ g of DNA by the application of Lipofectamine DNA coprecipitates in DME (GIBCO BRL). The medium was changed to serum-free DME at 3 h, and then the cells were cultured for another 12 or 36 h.

Cell synchronization

Mitotic HeLa cells were pooled by mitotic shake off as described in Piel et al. (2001). They were then replated on fibronectin- and collagen-coated glass coverslips. After 45 min, unattached cells were gently flushed in order to minimize desynchronization due to the variation of reattachment time of mitotic cells.

Cell fractionation

MDBK cells or siRNA-transfected HeLa cells were grown on plastic dishes for 72 h. Whole cell extract were obtained by direct resuspension of cells in boiling 1% SDS. After sonication, cells extracts were centrifuged 20 min at 100,000 g. The supernatants were further denatured in Laemmli sample buffer (25 mM Tris, 1% SDS, 10% glycerol, 1% 2-mercaptoethanol) and boiling.

Centrosome preparation

Centrosomes were purified from calf thymocytes as in Komesli et al. (1989). Centrosomes were isolated from KE 37 cells as described by Bornens et al. (1987).

Immunofluorescence microscopy

In all immunofluorescence experiments, antibodies were diluted in PBS supplemented with BSA at 1 mg/ml. Dilution factors are indicated in parentheses.

HeLa cell stably expressing GFP-centrin were grown on coverslips and washed in PBS before further processing. Cells were generally fixed for 4 min in methanol at -20°C , but for actin staining cells were fixed for 10 min in 3.7% paraformaldehyde and permeabilized 10 min with PBS containing 0.5% Triton X-100.

The preparations were subsequently washed in PBS, incubated with primary antibody mouse monoclonal anti- β -tubulin antibody clone 2-1 (1:100; Sigma-Aldrich), monoclonal anti-myc antibody (1:100; Invitrogen), rabbit polyclonal anti-protein N (1:500), or rabbit anti-peptide N1 to N9 (1:100) followed by the appropriate secondary antibody goat anti-mouse conjugated with fluorescein (1:250; Jackson ImmunoResearch Laboratories), goat anti-rabbit conjugated with rhodamine (1:250; Jackson ImmunoResearch Laboratories), rhodamine phalloidine (1:50; Molecular Probes), or goat anti-mouse conjugated with Alexa fluor 350 (1:500; Molecular Probes). Preparations were examined in Leica TCS-SP2 laser-scanning confocal microscope. Measures of intercentriolar distance and of cell perimeter to surface ratios were performed using IPLab software (Princeton Instruments).

Immuno EM

KE 37–isolated centrosomes were sedimented onto coverslips and processed as described in Chevrier et al. (1995) with the exception that tannic acid treatment of the fixed samples was omitted.

Immunoblot analysis

Gel electrophoresis analysis of each protein fraction was performed in one dimension according to Laemmli (1970). Proteins were transferred onto PVDF Immobilion membranes according to Towbin et al. (1979). The membrane was saturated in PBS 0.1% Tween 20 at room temperature for 1 h, and all subsequent washes and antibody dilutions were performed in this buffer. The membrane was incubated for 1 h with either rabbit polyclonal N antibody (1:5,000) or anti-peptide N(1–9) antibodies (1:500). After three 10-min washes, the membrane was incubated for 1 h with HRP goat anti-rabbit

(1:5,000; Biosource International), washed again, and then developed using a chemiluminescent ECL kit (Amersham Pharmacia Biotech).

Videomicroscopy

For time-lapse imaging, cells were plated on no. 1 1/2 coverslips. The coverslips were mounted in an opened rose chamber covered by a membrane permeable to CO₂. The microscope stage was equipped with a device enabling regulation of temperature and CO₂ level. Time-lapse Z sequences were collected on a Leica DMIRBE microscope controlled by Metamorph software (Universal Imaging Corp.). This microscope was equipped with a motorized stage, a 100× 1.4 NA Plan Apo Lens, and a cooled CCD camera (MicroMax 5 MHz; Roper Scientific). The final magnification on the camera chip was 134 nm/pixel (using the 2 × 2 binning mode of the camera). The illumination device was a 100-W halogen lamp with the potentiometer set under 8V to avoid overillumination. The acquisition time was 400 milliseconds. As a rule, 6–10 sequential z axis images were collected in 0.5-mm steps every 2 min. In these conditions, cells could enter mitosis and exit metaphase without any delay compared with cells recorded in phase-contrast only. Centriole tracking was performed automatically by Metamorph in maximal intensity projections computed from the original three-dimensional datasets.

Since Y-27632 is sensitive to light, cells were treated during 1 h with the drug before time-lapse recording, and the drug-containing medium was renewed at 1 h.

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