Polo-like kinase 1 regulates RhoA during cytokinesis exit in human cells

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Abstract. *Objective:* Both RhoA (Rho1) and polo-like kinase 1 (Plk1) are implicated in the regulation of cytokinesis, a cellular process that marks the division of cytoplasm of a parent cell into daughter cells after nuclear division. Cytokinesis failure is often accompanied by the generation of cells with an unstable tetraploid content, which predisposes it to chromosomal instability and oncogenic transformation. Several studies using lower eukaryotic systems demonstrate that RhoA and Plk1 are essential for mitotic progression and cytokinesis. *Materials and methods:* Physical and functional interactions between RhoA and Plk-1 were analyzed using subcellular localization of RhoA and Plk1 in HeLa cells by immunofluorescence and co-precipitation techniques, followed by Western blotting in RhoA transfected cells. *Results:* Plk1 localizes to kinetochores as well as to spindle poles during prophase and metaphase; it translocates to the midbody during telophase. RhoA is also enriched at the midbody region during telophase and colocalizes with Plk1. Recombinant RhoA, expressed as a GFP fusion protein, is enriched in the nucleus of HeLa and U2OS cells. Ectopically expressed GFP-RhoA does not cause significant cell death, although there exist a group of cells that appear to exhibit a delay in mitotic exit or in impaired cytokinesis. *Conclusion:* Co-immunoprecipitation reveals that RhoA and Plk1 physically interact and that their interaction appears to be enhanced during mitosis. Given the role of RhoA and Plk1 in cytokinesis, our findings suggest that regulated activation of RhoA is important for cytokinesis and that Plk1 may alter activation of RhoA during mitotic cytokinesis.

INTRODUCTION

To maintain the diploid content for a majority of somatic cells in higher animals, chromosome replication and segregation are tightly linked with successful cytokinesis at mitotic exit. Cytokinesis is the division of the cytoplasm of a parent cell into daughter cells after nuclear division (chromosome segregation), which is composed of a few distinct steps, such as central spindle assembly, cleavage furrow formation and final abscission of the midbody. Defects in cytokinesis or cytokinetic failure frequently causes formation of bi-nucleate or polyploid cells (Glutzer 2005). Aneuploidy, defined by numerical and structural abnormalities of chromosomes, is a

common phenomenon in malignant cells. In fact, proliferating transformed cells tend to become progressively aneuploid with frequency of divisions. Existing experimental evidence directly links impaired cytokinesis to oncogenic transformation (Fujiwara *et al.* 2005).

Extensive research in the past has identified candidate molecular components, including RhoA and polo-like kinase 1 (Plk1) that play a major role in regulating cytokinesis. Plk1 belongs to a family of conserved regulators of multiple events during cell division. Vertebrate cells contain four proteins (Plk1, Plk2, Plk3 and Plk4) that exhibit marked sequence homology to Polo, the founding member of the polo-like kinase family (Barr *et al.* 2004). Plks share a highly conserved protein kinase domain in the amino-terminal region of these proteins. The kinase activity of Plks is critical for their various cellular functions. Plks also contain common structural motifs called polo boxes in the non-catalytic carboxyl-terminal region (Barr *et al.* 2004). Recent studies have shown that the polo box domain functions as a docking site for various serine/threonine-phosphorylated proteins (Elia *et al.* 2003). Several recent studies show that Plk1 and its orthologues are important regulators for cytokinesis (Barr *et al.* 2004).

A second family of proteins that is involved in regulating cytokinesis that of the Rho GTPases (Narumiya & Yasuda 2006). These proteins alternate between the inactive GDP-bound form and the active GTP-bound form. Several recent studies indicate that RhoA (also termed Rho1) plays a significant role in controlling cytokinesis (Glotzer 2005; Hickson *et al.* 2006). During late mitosis (anaphase), spindle microtubules become bundled to form the central spindle that is essential for the positioning of the cleavage furrow, the indentation of cell surface membrane that marks initiation of the process of cytoplasmic cleavage. RhoA is known to be involved in regulating cleavage furrow positioning and subsequent events associated with cytokinesis (Glotzer 2005).

Several lines of evidence suggest that there exists a regulatory relationship between RhoA and Plk1 during cytokinesis, for example, RhoA activity is stimulated by Rho exchange factors; ECT2 (a Rho exchange factor) interacts with Plk1 after priming by phosphorylation by Cdk1 (Niya *et al.* 2006). In addition, polo-like kinase Cdc5 in budding yeast controls subcellular localization and activation of RhoA at the division site (Yoshida *et al.* 2006). Given the importance of Plk1 and RhoA in mitosis, we have hypothesized that RhoA and Plk1 may physically and functionally interact during the mammalian cell cycle. Our initial studies have shown that both RhoA and Plk1 localize to the midbody at telophase. Co-immunoprecipitation analysis reveals that these two proteins physically interact and that their interaction appears to be enhanced during mitosis.

MATERIALS AND METHODS

Cell culture

HeLa and U2OS cell lines were obtained from American Type Culture Collection. These cells were cultured under 5% CO₂ at 37 °C in Dulbecco's minimum essential medium (DMEM), 15% foetal bovine serum (FBS) and antibiotics (100 µg/mL penicillin, 50 µg/mL streptomycin sulphate). Cell cultures were free of mycoplasma contamination as tested routinely in our laboratory.

RhoA expression construct

Human RhoA cDNA clone (MGC-14663) was obtained from American Type Culture Collection. The RhoA cDNA insert was amplified with DNA primers as follows: the forward primer, 5'-AATGGCTGCCATCCGGAAGAA-3', and the reverse primer, 5'-CAAGACAAGGCACCCAGATT-3'. The amplified RhoA cDNA, after cutting with restriction enzymes BamH1 and EcoR1, was cloned into a pGFP-N3 plasmid at the EcoR1 and BamH1 sites. The fusion site between RhoA

cDNA and the DNA sequence coding for green fluorescence protein was confirmed by direct DNA sequencing.

Fluorescence microscopy

HeLa and U2OS cells cultured in chamber slides over night were transfected with or without pGFP-RhoA for 24 h or 48 h using the Lipofectamine approach. Expression of GFP-RhoA was directly monitored through the usage of fluorescence microscopy. HeLa cells transfected with or without plasmid DNA were fixed in 4% paraformaldehyde and were treated with 0.1% Triton X-100 on ice, followed by washing three times with phosphate-buffered saline (PBS). After blocking with 2.0% bovine serum albumin in PBS for 15 min, cells were incubated for 1 h with antibodies to RhoA, Plk1 or α -tubulin, they were then washed with PBS and were incubated with appropriate secondary antibodies conjugated with Rhodamine Red-X or FITC (Jackson Immuno Research, West Grove, PA, USA). Cells were finally stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/mL, Fluka, Germany). Fluorescence microscopy was performed using a Nikon microscope, and images were captured by a digital camera equipped with the appropriate imaging software.

Western blot analysis

HeLa cells were transfected with or without pGFP-RhoA plasmids for 24 h. Cells were then collected and lysed. Lysates were centrifuged at $12\,000 \times g$ for 10 min at 4 °C, and supernatants were collected. Equal amounts of proteins were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electro-transfer to nylon membranes. Protein blots were probed with anti-GFP antibody. Specific signals were detected using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Sigma, St. Louis, MO, USA) and were enhanced with chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Immunoprecipitation

HeLa cells were cultured in the presence or absence of nocodazole (0.5 g/mL) for 16 h. Lysates were prepared from mitotic, as well as from interphase cells. After pre-clearing with protein A/G beads (Santa Cruz, CA, USA), protein lysates of interphase and mitotic stages (1 mg each) were incubated with anti-Plk1 antibody (5 mg) overnight at 4 °C. Protein A/G beads were then added to the lysates and the incubation was continued at room temperature for 1 h. After extensive washing with lysis buffer, protein A/G beads were suspended in SDS-PAGE sample buffer for subsequent analysis.

RESULTS

Both RhoA and Plk1 are important regulators of mitotic exit. As the first step to determine whether these two proteins may regulate each other, we examined subcellular localization of Plk1 and RhoA in mitotic cells. Fluorescence microscopy revealed that in prophase cells Plk1 was localized at spindle poles, although strong Plk1 signals were also detected in kinetochores. During metaphase, a significant amount of Plk1 remained at both spindle poles and kinetochores, although diffused signals were detected along mitotic spindles (Fig. 1a). During telophase, strong Plk1 staining was detected at the midbody. However, while RhoA exhibited no discrete subcellular localization patterns in other stages of the cell cycle (data not shown) it was concentrated at the division site (the midbody), thus being in a close proximity with Plk1 (Fig. 1b).

Figure 1. Subcellular localization of Plk1 and RhoA during mitosis. (a) HeLa cells were fixed and stained with antibodies to Plk1 and α -tubulin. DNA was stained with DAPI. The stained cells were then examined by fluorescence microscopy. Representative prophase and metaphase cells are shown. Arrows indicate the positions of spindle poles. (b) HeLa cells were fixed and stained with antibodies to RhoA and Plk1. DNA was stained with DAPI. A representative telophase cell was shown. Arrow indicates the position of the midbody.

To determine the role of RhoA in regulating cytokinesis, we made an expression construct through in-frame fusion of RhoA cDNA with the DNA sequence encoding green fluorescence protein (Fig. 2a). The cloned RhoA cDNA insert was confirmed by digestion of pGFP-RhoA plasmid DNA with *Eco*R1 and *Bam*H1 followed by agarose gel electrophoresis. As expected, an insert of about 650 base pairs was released after the digestion, and this fragment was not present in samples containing no restriction endonucleases (Fig. 2b). Direct DNA sequencing also confirmed that RhoA cDNA was fused in-frame with GFP DNA (data not shown). We then introduced pGFP-RhoA plasmids into U2OS and HeLa cells through transfection. GFP-RhoA was efficiently expressed in both U2OS and HeLa cells (Fig. 3a), which was also confirmed by indirect fluorescence microscopy after staining with the antibody to GFP (Fig. 3b). Moreover, a strong protein band of about 50 kDa was detected in HeLa cells transfected with pGFP-RhoA,

Figure 2. Cloning of RhoA cDNA into pGFP plasmid. (a) RhoA cDNA amplified using a pair of primers was cloned at BamH1 and EcoR1 sites of pGFP-N3 plasmid. (b) Plasmid DNA of pGFP-RhoA digested with *Bam*H1 and *Eco*R1 was analysed by agarose gel electrophoresis.

but not in untransfected control cells (Fig. 4a). Some minor bands were also detected in cells transfected with pGFP-RhoA, but most likely these were proteolytic cleavage products.

GFP-RhoA was enriched in the nucleus, although cytoplasmic GFP-RhoA was also detected (Fig. 3a). No significant cell death was observed in cells ectopically expressing GFP-RhoA. On the other hand, a fraction of cells expressing GFP-RhoA apparently underwent a delay in mitotic exit, resulting in enrichment of doublet cells (Fig. 4b). Configuration of cells with the appearance of their connection with each other (Fig. 5b) suggested that over-expression of RhoA may adversely affect completion of cytokinesis. We then performed co-immunoprecipitation experiments with which physical interaction between RhoA and Plk1 was examined. Equal amounts of interphase

Figure 3. Expression of GFP-RhoA. (a) U2OS and HeLa cells were transfected with pGFP-RhoA expression plasmid for 48 h. Transfected cells were directly examined by fluorescence microscopy. Representative images are shown. No fluorescence was detected with parental cells (data not shown). (b) HeLa cells transfected with pGFP-RhoA for 24 h were fixed and stained with the antibody to GFP. DNA was stained with DAPI. An anaphase cell and an interphase cell expressing no transfected protein are also shown (arrows).

Figure 4. Analysis of cells ectopically expressing GFP-RhoA. (a) HeLa cells transfected with or without pGFP-RhoA for 48 h was lysed. Equal amounts of proteins were blotted for GFP-RhoA and β -actin. (b) HeLa cells expressing transfected GFP-RhoA were directly examined for their morphology using fluorescence microscopy. Cells with apparent defects in cytokinesis are shown.

Figure 5. RhoA interacts with Plk1. Interphase and mitotic cells were collected and lysed. Equal amounts (1 mg) of proteins were immunoprecipitated with anti-Plk1 antibody or with a control antibody (CNTL IgG). After overnight incubation, immunoprecipitates were blotted for Plk1 and RhoA. The relative positions of IgGs were also indicated.

and mitotic HeLa cell lysates were immunoprecipitated with Plk1 IgG or with control IgG. After overnight incubation, immunoprecipitates were collected and analysed by SDS-PAGE followed by immunoblotting for RhoA, as well as for Plk1. We observed that Plk1 antibody, but not the control IgG, was capable of pulling down RhoA antigen; more RhoA signals were immunoprecipitated by Plk1 antibody when mitotic lysates were used (Fig. 5).

DISCUSSION

The cytoskeleton undergoes tremendous remodeling during mitotic progression and mitotic exit. RhoA and Plk1 are involved in regulating cytoskeleton molecules, including actin and microtubules. Our current study is consistent with the proposed function that RhoA and Plk1 may physically and functionally interact during late mitosis.

- 1 Both RhoA and Plk1 localize to the midbody region during telophase in HeLa cells.
- 2 Ectopic expression of RhoA partially impairs cytokinesis.
- 3 Plk1 physically interacts with RhoA and its interaction with RhoA is enhanced during mitosis.

Two major questions regarding their regulatory relationship remain unanswered. First, it is unclear whether the interaction between RhoA and Plk1 is direct or is via other components. A recent study shows that Plk1 phosphorylates ECT2 at G₂/M transition (Niiya *et al.* 2006). ECT2 is a Rho exchange factor that activates RhoA. Given that ECT2 is also involved in regulating cytokinesis, it is possible that the interaction between RhoA and Plk1 is mediated through ECT2 or a related protein. Second, it remains unclear whether RhoA regulates Plk1 activity or vice versa. Rho protein is known to activate downstream effector molecules that include protein kinases (Loirand *et al.* 2006). On the other hand, a recent study shows that Cdc5 controls the activation of Rho1 at the division site in budding yeast, indicating that the yeast polo-like kinase is upstream of RhoA in the signalling pathway that controls cytokinesis. Therefore, additional

studies are needed to better clarify the regulatory hierarchy between RhoA and Plk1 in mammalian cells.

Many therapeutic agents used in the clinic for various human malignancies are compounds that disrupt mitotic microtubules (e.g. taxol). Thus, it is tempting to speculate that the effect of taxol on cancer cells may be mediated through disruption of mitotic processes, including cytokinesis due to defects in microtubule dynamics. Further study of molecular mechanisms by which cells control cytokinesis regulated by RhoA and Plk1 may enable us to discover more powerful therapeutic compounds and achieve more specificity for targeting tumour cells for programmed cell death. It is anticipated that such new compounds can take advantage of differences between cytokinesis control in normal and cancer cells, thereby enhancing their therapeutic efficacy.

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