During cytokinesis the actomyosin-based contractile ring is formed at the equator, constricted, and then disassembled prior to cell abscission. Coflin stimulates actin filament disassembly and is implicated in the regulation of contractile ring dynamics. However, little is known about the mechanism controlling coflin activity during cytokinesis. Cofilin is inactivated by phosphorylation on Ser-3 by LIM-kinase-1 (LIMK1) and is reactivated by a protein phosphatase Slingshot-1 (SSH1). Here we show that the phosphatase activity of SSH1 decreases in the early stages of mitosis and is elevated in telophase and cytokinesis in HeLa cells, a time course correlating with that of coflin dephosphorylation. SSH1 co-localizes with F-actin and accumulates onto the cleavage furrow and the midbody. Expression of a phosphatase-inactive SSH1 induces aberrant accumulation of F-actin and phospho-cofilin near the midbody in the final stage of cytokinesis and frequently leads to the regression of the cleavage furrow and the formation of multinucleate cells. Co-expression of coflin rescued the inhibitory effect of phosphatase-inactive SSH1 on cytokinesis. These results suggest that SSH1 plays a critical role in cytokinesis by dephosphorylating and reactivating coflin in later stages of mitosis.

Actin filament dynamics and reorganization play a principal role in cytokinesis in animal cells (1–3). During cytokinesis actin filaments are dynamically reconstructed and concentrated onto the cleavage furrow, and an actomyosin-based contractile ring is formed at the equator of dividing cells, is constricted, and then disappears at the end of cytokinesis (1–3). Cofilin and its closely related protein, actin depolymerizing factor (ADF),1 are key regulators of actin filament dynamics and reorganization by stimulating depolymerization and severance of actin filaments (4–6). Cofilin/ADF (hereafter referred to as coflin) localizes at the cleavage furrow and the midbody during cytokinesis in cultured mammalian cells and in Xenopus fertilized eggs (7, 8). Decreases in coflin expression or activity by gene mutation, RNA interference, or antibody injection caused frequent failures in cytokinesis, therefore indicating that coflin plays a critical role in processes related to cytokinesis (8–10). However, little is known of mechanisms controlling coflin activity during cytokinesis.

Cofilin activity is negatively regulated by phosphorylation at Ser-3 by LIM-kinase-1 (LIMK1) (11, 12) and reactivated by a protein phosphatase Slingshot-1 (SSH1) (13). We earlier reported that the level of the Ser-3-phosphorylated coflin (P-cofilin) periodically changes during the cell division cycle in HeLa cells; it increases in early stages of mitosis (prometaphase and metaphase), then is gradually dephosphorylated in telophase and cytokinesis (14). As coflin activity is negatively regulated by phosphorylation at Ser-3 (15, 16), this means that coflin is inactivated in the early stages of mitosis and reactivated in the later stages. The kinase activity of LIMK1 was shown to increase in the early stages of mitosis and then decline to the basal level in later stages (14, 17). Thus, LIMK1 seems to be involved in changes in coflin activity during the cell cycle.

Dephosphorylation of coflin could be caused by down-regulation of coflin kinases, up-regulation of coflin phosphatases, or both. SSH1 is a member of SSH family phosphatases that specifically dephosphorylate P-cofilin in vitro and in vivo (13, 18). When we examined changes in the phosphatase activity of SSH1 during the cell cycle, we found that this activity decreases in early stages of mitosis and increases in later stages, a time course correlating with that of the level of coflin dephosphorylation. SSH1 co-localizes with F-actin and accumulates onto the cleavage furrow and the midbody during cytokinesis. We also found that expression of a phosphatase-inactive mutant of SSH1 increases the level of P-cofilin throughout the cell cycle, induces aberrant accumulation of F-actin near the midbody in the final stage of cytokinesis, and leads to frequent formation of multinucleate cells. We propose that SSH1 plays a critical role for cytokinesis in animal cells by dephosphorylating and reactivating coflin.

EXPERIMENTAL PROCEDURES

**Plasmid Construction**—To construct a plasmid for yellow fluorescent protein (YFP)-tagged wild-type (WT) SSH1, hSSH1L cDNA in pCDNA3.1/Myc-His(+) vector (13) was cut and subcloned into pEYFP-C1 (Clontech, Palo Alto, CA). A plasmid for YFP-SSH1(CS) with a replacement of Cys-393 by serine was generated by PCR-based mutagenesis. Plasmid for cyan fluorescent protein (CFP)-tagged histone H2B was generated by subcloning the PCR-amplified mouse histone H2B cDNA into pECPF-C1 (Clontech). Plasmids coding for Myc-SSH1(WT), Myc-SSH1(CS), CFP-LIMK1, YFP-cofilin(WT), YFP-

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1 The abbreviations used are: ADF, actin depolymerizing factor; CFP, cyan fluorescent protein; DAPI, 4,6-diamidino-2-phenylindole; LIMK1, LIM-kinase-1; P-cofilin, Ser-3-phosphorylated coflin; SSH1, Slingshot-1; SSH1(CS), a Slingshot-1 mutant in which catalytic Cys is replaced by Ser; WT, wild-type; YFP, yellow fluorescent protein.
cofilin (S3A), and YFP-cofilin (S3E) were constructed as described previously (13, 14, 16).

Antibodies—Rabbit anti-SSH1 antibody (AB2271) was raised against the C-terminal peptide (GGCPAPENLKSWSMSKS) of human SSH1. Anti-P-cofilin antibody specific to the Ser-3-phosphorylated form of cofilin was prepared as described previously (19). Anti-Myc monoclonal antibody (9E10) was purchased from Roche Diagnostics.

Cell Culture Experiments—HeLa cells were synchronized at the beginning of the S phase by double thymidine block and released for 6–12 h as described previously (14, 20). The cell cycle stage was determined by flow cytometry (14). HeLa cells were arrested at early mitotic phase by nocodazole, and mitotic and interphase cells were collected selectively by the mechanical shake-off procedure (14, 20). Mitotic cells were released from nocodazole arrest for 0–180 min, and the cell cycle state was monitored by staining with 4,6-diamidino-2-phenylindole (DAPI) and anti-tubulin monoclonal antibody (9E10) was purchased from Roche Diagnostics. Anti-P-cofilin antibody specific to the Ser-3-phosphorylated form of human cofilin, or anti-Myc antibody. DAPI was used for DNA staining. After washing with phosphate-buffered saline, coverslips were mounted on a glass slide and images were obtained using a fluorescence microscope (model DMLB, Leica, Nussloch, Germany).

Time-lapse Fluorescence Analysis—For time-lapse imaging, HeLa cells were plated on a 35-mm glass-bottom dish and co-transfected with plasmids for YFP-fused protein and CFP-H2B. These cell lines were observed using an inverted fluorescence microscope (model DMIREB, Leica), equipped with an Apochromat 63× oil objective (numerical aperture, 1.32) lens and a YFP- or CFP-optimized filter set (Omega Optical, Brattleboro, VT). Time-lapse fluorescence images were captured every 3–10 min for 120–180 min with 50–100 ms exposures, using a Coolscope HQ-cooled charge-coupled device camera (1024 × 1024 pixels, Roper Scientific, Wetzlar, Germany) driven by a Q500FW Imaging Software (Leica). The room temperature was maintained at 37 °C.

RESULTS

Cell Cycle-associated Changes in the Phosphatase Activity of SSH1—To investigate the role of SSH1 during the cell cycle, we measured the phosphatase activity of SSH1 in different stages of the cell cycle. HeLa cells were synchronized by the beginning of the S phase by double thymidine block. At different times after release from thymidine block, endogenous SSH1 was immunoprecipitated and subjected to in vitro phosphatase assay and immunoblot analysis (Fig. 1A). Cells at 6, 9, and 12 h after release were mainly in the S, G2/M, and G1 phases, respectively, as assessed by flow cytometry analysis (data not shown and Ref. 14). The phosphatase activity of SSH1 decreased in cells at 6 and 9 h and then increased in cells at 12 h (Fig. 1A, bottom panel). Thus, the phosphatase activity of SSH1 depends on the cell cycle, the activity being minimal in the G1/M phase. Immunoblot analysis revealed three separate anti-SSH1 immunoreactive bands of apparent molecular mass around 150–170 kDa (Fig. 1A, top panel). A slow-migrating band significantly increased in cells at 9 h, whereas two fast-migrating bands were prominent in cells arrested at the beginning of the S phase (at zero time) and in asynchronous cells, which suggests that the activity may be regulated by phosphorylation (see Fig. 1C).

We next analyzed changes in the phosphatase activity of SSH1 during mitosis. HeLa cells were synchronized at the early mitotic phase by nocodazole and released for 0–180 min to allow for cell cycle progression. Cells at 0, 45, 90, and 180 min after release were mainly in prometaphase, metaphase, telophase, and G1 phase, respectively, as monitored by staining for tubulin and DNA (see “Experimental Procedures”). The activity of SSH1 in cells at 0 and 45 min was significantly decreased, compared with that in interphase (I) cells then gradually increased and almost reverted to the initial level at 180 min (Fig. 1B, bottom panel). Immunoblot analysis revealed that a slow-migrating form of SSH1 was prominent in cells at 0, 45, and 90 min after release, although total amounts of SSH1 remained unchanged throughout the cell cycle (Fig. 1B, top panel). Treatment of interphase and mitotic SSH1 with calf intestinal alkaline phosphatase abolished the upper gel mobility shift (Fig. 1C), indicating that slow-migrating bands are the phosphorylated forms of SSH1. The upper mobility shift of SSH1 correlated well with decrease in the phosphatase activity (Fig. 1, A and B). These results indicate that SSH1 is phosho-
rulated and inactivated in early stages of mitosis (prometaphase and metaphase) then dephosphorylated and reactivated in later stages (telophase and cytokinesis).

Fig. 1D summarizes changes in the phosphatase activity of SSH1 during the cell cycle, compared with the previous data on changes in the level of P-cofilin and the kinase activity of LIMK1 (14). The phosphatase activity of SSH1 and the kinase activity of LIMK1 are almost inversely regulated during the cell cycle. The level of P-cofilin correlates well with changes in LIMK1 and SSH1 activities.

**SSH1 Localizes onto the Cleavage Furrow, and Expression of SSH1(CS) Induces Aberrant Accumulation of F-actin**—To determine the subcellular localization of SSH1 during mitosis, YFP-tagged wild-type SSH1 (YFP-SSH1(WT)) or its phosphatase-inactive mutant (YFP-SSH1(CS)), in which the catalytic cysteine residue (Cys-393) is replaced by serine, was expressed in HeLa cells, and the cells were synchronized and stained with rhodamine-phalloidin for F-actin and DAPI for DNA (Fig. 2).

Whereas control YFP was distributed diffusely in the cytoplasm during mitosis (Fig. 2, a–d), both YFP-SSH1(WT) and YFP-SSH1(CS) co-localized with F-actin (Fig. 2, e–l), in accord with their potential to bind to F-actin in vitro (13); they were uniformly distributed on the cortical actin in metaphase (Fig. 2, e and i) and concentrated onto the cleavage furrow in anaphase and telophase (Fig. 2, f, g, j, and k) and on the intercellular bridge (midbody) in late telophase (Fig. 2, h and l). In cells expressing YFP-SSH1(WT) or YFP-SSH1(CS), the cleavage furrow was normally formed and constricted during anaphase to telophase. In late telophase, however, expression of YFP-SSH1(CS) induced aberrant F-actin accumulation near the midbody (Fig. 2l), whereas no apparent effect was observed in cells expressing YFP-SSH1(WT) (Fig. 2h). YFP-SSH1(CS) co-localized with these aberrant F-actin aggregates (Fig. 2l). Thus, SSH1 localizes on the cleavage furrow and midbody during cytokinesis and seems to play a critical role in the regulation of actin filament dynamics in the terminal stage of cytokinesis.

**Expression of SSH1(CS) Increases the Level of P-cofilin**—To further examine the role of SSH1 in the regulation of actin filament dynamics during mitosis and cytokinesis, we analyzed the P-cofilin level in cells expressing YFP-SSH1(WT) or YFP-SSH1(CS) by immunostaining using an anti-P-cofilin antibody that specifically recognizes P-cofilin (19) (Fig. 3). Consistent with our previous observations (14), in cells expressing control YFP, significant increases in the P-cofilin level were observed in metaphase (Fig. 3a) and anaphase (Fig. 3b) but not in telophase (Fig. 3c), compared with findings in surrounding interphase cells. Expression of YFP-SSH1(WT) lowered the P-cofilin level in metaphase and anaphase, perhaps because of its cofilin-phosphatase activity (Fig. 3, d–f). In cells expressing YFP-SSH1(CS), the high level of P-cofilin was retained from metaphase to telophase, and P-cofilin accumulated on the cleavage furrow and intercellular bridge, both being sites rich in F-actin and YFP-SSH1(CS) (Fig. 3, g–j). This is in contrast to the pattern seen in control YFP-expressing cells, in which P-cofilin is diffusely distributed in the cytoplasm (Fig. 3, a–c). These results suggest that SSH1(CS) functions as a dominant-negative form and suppresses cofilin dephosphorylation in later stages of mitosis.

**Expression of SSH1(CS) Increases the Ratio of Multinucleate Cells**—We next examined effects of overexpression of SSH1 or its CS mutant on cytokinesis. HeLa cells transfected with Myc-tagged SSH1(WT) or SSH1(CS) were cultured for 60 h (a period sufficient for one or two cycles of cell division) and stained with DAPI (Fig. 4A). About 38% of cells expressing Myc-SSH1(CS) became multinucleate, whereas only a small percentage

![Fig. 2. Co-localization of SSH1 with F-actin during mitosis and cytokinesis and aberrant accumulation of F-actin by expression of SSH1(CS).](image-url)
expression of Myc-SSH1(WT) or control YFP exhibited such multinucleate phenotypes (Fig. 4, A and B). No apparent effect on cytokinesis was observed in cells expressing cofilin(WT) or its S3A (non-phosphorylatable) or S3E (phosphorylation-mimic) mutant, in which Ser-3 was replaced by alanine or glutamic acid (Fig. 4B). Importantly, co-expression of cofilin(WT) with SSH1(CS) significantly reduced the ratio of multinucleate cells (Fig. 4, A and B), which suggests that SSH1(CS) induces multinucleate cells by suppressing dephosphorylation/activation of cofilin. As reported previously (14), expression of LIMK1(WT) induced the formation of multinucleate cells (Fig. 4, A and B). This effect was partially overcome by co-expression of non-phosphorylatable cofilin(S3A), but not with cofilin(WT) or cofilin(S3E) (Fig. 4, A and B), which suggests that LIMK1 induces multinucleate cells by phosphorylating and inactivating cofilin. Co-expression of SSH1(WT) with LIMK1 significantly reduced the ratio of multinucleate cells induced by LIMK1, which further suggests that SSH1 plays a role in cytokinesis by dephosphorylating cofilin. Co-expression of SSH1(CS) with LIMK1 synergistically enhanced the ratio of multinucleate cells.

Expression of SSH1(CS) Induces the Regression of the Cleavage Furrow—Appearance of multinucleate cells suggests a failure of cytokinesis in cells expressing SSH1(CS). To better understand how SSH1(CS) blocks cytokinesis, the processes of cytokinesis of HeLa cells expressing YFP, YFP-SSH1(WT), or YFP-SSH1(CS) were monitored, using time-lapse fluorescence microscopy. CFP-fused histone H2B (CFP-H2B) was co-expressed to visualize chromosomes. Cells expressing control

SSH1(CS) induces multinucleate cells by suppressing dephosphorylation/activation of cofilin. As reported previously (14), expression of LIMK1(WT) induced the formation of multinucleate cells (Fig. 4, A and B). This effect was partially overcome by co-expression of non-phosphorylatable cofilin(S3A), but not with cofilin(WT) or cofilin(S3E) (Fig. 4, A and B), which suggests that LIMK1 induces multinucleate cells by phosphorylating and inactivating cofilin. Co-expression of SSH1(WT) with LIMK1 significantly reduced the ratio of multinucleate cells induced by LIMK1, which further suggests that SSH1 plays a role in cytokinesis by dephosphorylating cofilin. Co-expression of SSH1(CS) with LIMK1 synergistically enhanced the ratio of multinucleate cells.
YFP or YFP-SSH1(WT) normally divided and separated into two daughter cells (Fig. 5, A and B). In contrast, in cells expressing YFP-SSH1(CS), the cleavage furrow was formed and ingressed normally, but at the final stage of cytokinesis the daughter cells could not separate, and the furrow gradually regressed to produce binucleate cells (Fig. 5 C). Of seven YFP-SSH1(CS)-expressing cells observed by time-laps analysis, four exhibited the regression of the cleavage furrow. YFP-SSH1(WT) was concentrated on the intercellular bridge at the terminal stage of cytokinesis but diffusely distributed into the cortex of two daughter cells, as the cells spread after cell division (Fig. 5 B). In contrast, YFP-SSH1(CS) remained localized on the position of intercellular bridge even after the furrow regressed and cells spread (Fig. 5 C). These results suggest that SSH1(CS) blocks cytokinesis by suppressing cell abscission at the final stage of cytokinesis.

DISCUSSION

In the present study, we found that the phosphatase activity of SSH1 changes significantly during the cell cycle; it decreases in early stages of mitosis (prometaphase and metaphase) and is elevated in later stages (telophase and cytokinesis). The kinase activity of LIMK1 is almost inversely regulated during the cell cycle; it increases in early stages of mitosis and decreases in later stages (14). As summarized in Fig. 1D, cell cycle-associated changes in the level of P-cofilin (14) correlate well with changes in LIMK1 and SSH1 activities, which suggests that cofilin activity is regulated during the cell cycle by phosphorylation and dephosphorylation through changes in both LIMK1 and SSH1 activities. It is probable that activation of LIMK1 and inactivation of SSH1 are involved in phosphorylation and inactivation of cofilin in early stages of mitosis, whereas activation of SSH1 and inactivation of LIMK1 are related to dephosphorylation and reactivation of cofilin in later stages of mitosis.

Ectopic expression of LIMK1 (14) or phosphatase-inactive SSH1(CS) (in this study) increased the level of cofilin phosphorylation throughout the cell cycle and induced the formation of multinucleate cells. Co-expression of cofilin(S3A) with LIMK1 or co-expression of cofilin(WT) with SSH1(CS) significantly reduced the ratio of multinucleate cells. Together with the observation that the P-cofilin level was retained at the high level throughout the cell cycle in cells expressing LIMK1 (14) or SSH1(CS) (Fig. 3, g–j), these findings suggest that the production of multinucleate cells by LIMK1 or SSH1(CS) is caused by insufficient dephosphorylation/reactivation of cofilin in later stages of mitosis and that dephosphorylation and reactivation of cofilin in later stages of mitosis is critical for cytokinesis. Co-expression of SSH1(WT) reversed the effect of LIMK1 on multinucleate cell formation, which indicates that SSH1 has the potential to neutralize the inhibitory effect of LIMK1 on cytokinesis by dephosphorylating cofilin. SSH1(WT) and SSH1(CS) co-localize with F-actin and are concentrated onto the cleavage furrow and midbody during cytokinesis. SSH1(CS) probably suppresses cofilin dephosphorylation by competing with endogenous SSH1 for localization on the cleavage furrow and/or activation by upstream regulators in a dominant-negative manner. Co-expression of cofilin(WT) did not rescue the effect of LIMK1 overexpression, probably because ectopic LIMK1 phosphorylates and inactivates cofilin(WT) co-expressed. Expression of cofilin(S3E), which mimics P-cofilin, had no effect on cytokinesis, because it does not bind to either G- or F-actin and has no activity to depolymerize and sever actin filaments (15, 16).

In contrast to the effects of expression of LIMK1 or SSH1(CS), expression of wild-type SSH1 significantly reduced the P-cofilin level in early stages of mitosis, compared with that in cells expressing control YFP (Fig. 3), but exhibited no apparent effect on cytokinesis, as assessed by counting the ratio of multinucleate cells (Fig. 4) and by time-lapse scanning with fluorescence microscopy (Fig. 5). Similarly, expression of cofilin(WT) or cofilin(S3A) did not significantly affect the ratio of multinucleate cells (Fig. 4). Thus, when cells were treated to

![Fig. 5. Time-lapse analysis of cytokinesis of living cells expressing SSH1(WT) or SSH1(CS).](image-url)
increase cofilin activity during the cell cycle, we observed no apparent effect on cytokinesis. In contrast, Abe et al. (8) reported that injection of glutathione S-transferase-coupled Xenopus ADF/cofilin (a non-phosphorylatable and constitutively active form of Xenopus ADF/cofilin) into Xenopus embryos significantly blocked cytokinesis. The apparent contradiction may be due to the differences in experimental systems (cultured mammalian cells versus Xenopus eggs, and plasmid transfection versus microinjection) and proteins overexpressed (S3A-cofilin versus glutathione S-transferase-coupled Xenopus ADF/cofilin). Further studies will be required to explain such contradictory results and to better understand the physiological importance of the increase in P-cofilin levels in early stages of mitosis.

The contractile ring is a dynamic structure in which actin filaments continuously assemble and disassemble (1–3). Recent studies revealed that the Arp2/3 complex, formins and profilin are required for actin polymerization to maintain the contractile ring during cytokinesis as well as for initial ring formation (21, 22). Cofilin seems to play a critical role in regulating dynamics of the contractile ring by depolymerizing and severing actin filaments. Decreases in cofilin expression by gene mutation or RNA interference in Drosophila and activated in early stages of mitosis and dephosphorylated in later stages of mitosis (14). Although Rho-associated kinase (ROCK) and p21-activated kinase (PAK) phosphorylate and activate LIMK1 (23–25), several lines of evidence suggest that these kinases are not related to mitotic phosphorylation and activation of LIMK1 (14). Identification of kinase(s) and phosphatase(s) responsible for the regulation of SSH1 and LIMK1 activities during the cell cycle will be important to understand the signaling mechanisms that link the activity of cell cycle regulators to contractile ring dynamics and to elucidate mechanisms governing how cell cleavage is temporally and spatially regulated.

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