Src Signaling Regulates Completion of Abscission in Cytokinesis through ERK/MAPK Activation at the Midbody*

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Src family non-receptor-type tyrosine kinases regulate a wide variety of cellular events including cell cycle progression in G₂/M phase. Here, we show that Src signaling regulates the terminal step in cytokinesis called abscission in HeLa cells. Abscission failure with an unusually elongated intercellular bridge containing the midbody is induced by treatment with the chemical Src inhibitors PP2 and SU6656 or expression of membrane-anchored Csk chimeras. By anti-phosphotyrosine immunofluorescence and live cell imaging, completion of abscission requires Src-mediated tyrosine phosphorylation during early stages of mitosis (before cleavage furrow formation), which is subsequently delivered to the midbody through Rab11-driven vesicle transport. Treatment with U0126, a MEK inhibitor, decreases tyrosine phosphorylation levels at the midbody, leading to abscission failure. Activated ERK by MEK-catalyzed dual phosphorylation on threonine and tyrosine residues in the TEY sequence, which is strongly detected by anti-phosphotyrosine antibody, is transported to the midbody in a Rab11-dependent manner. Src kinase activity during the early mitosis mediates ERK activation in late cytokinesis, indicating that Src-mediated signaling for abscission is spatially and temporally transmitted. Thus, these results suggest that recruitment of activated ERK, which is phosphorylated by MEK downstream of Src kinases, to the midbody plays an important role in completion of abscission.

Cytokinesis is the last stage of mitosis when a single cell divides into two daughter cells after chromosome segregation. Defects in cytokinesis can induce cell death and genetic instability that brings about the development of aneuploid malignancies (1). The process of cytokinesis must be spatially and temporally controlled in a sophisticated manner. During cytokinesis, two daughter cells are connected in a pair with a cytoplasmic bridge containing the midbody, which consists of bundled anti-parallel microtubules and associated proteins. The overlapped region of plus ends of microtubules forms a dense structure called the midbody matrix (2, 3). These microtubule-based structures play key roles in cytokinesis from initiation to completion.

Membrane trafficking ceases during early stages of mitosis, however, it resumes at late mitosis: internalized vesicles are trafficked to the recycling endosome during cleavage furrow ingression, and subsequently to the midbody during late stages of cytokinesis (4). There is accumulating evidence that coordinated membrane trafficking to the cleavage furrow and the midbody is essential for completion of cytokinesis (2–10). For instance, inhibition of membrane transport via recycling endosomes (by expression of small interfering RNA for Rab11 or dominant-negative Rab11) or dispersion of the Golgi apparatus (by brefeldin A) causes defects in the late stage of cytokinesis in both Caenorhabditis elegans and mammalian cells (7–9). However, signal transduction mediated via recycling endosomes at the midbody is unknown.

Src family kinases (SFKs),¹ which belong to a family of non-receptor-type protein-tyrosine kinases, are activated by various stimuli, and are involved in a wide range of signaling events, such as proliferation, migration, and cytoskeletal reorganization (11). SFKs are localized to membranes through their N-terminal myristoylation (11). The catalytic activity of SFKs is suppressed by phosphorylation of their C-terminal tyrosine residues by Csk family tyrosine kinases (11–17). Efficient SFK inhibition is induced by Csk translocation from the cytosol to the plasma membrane (14–16).

Previous studies showed that the catalytic activity of SFKs is required for mitotic progression, because G₂/M progression is prevented by microinjection of a neutralizing anti-Src/Fyn/Yes antibody or the SH2 domain of Fyn (18), and by treatment with PD173955, a chemical inhibitor of SFKs (19). The regulation of SFK activity in mitosis is dependent on dephosphorylation of their C-terminal tyrosine residues because c-Src is not activated during mitosis in protein-tyrosine phosphatase α knockout cells (20). The activity of c-Src and accessibility of its SH2 domain for protein binding are increased at the initial phase of mitosis (19, 21–23), suggesting that SFKs may play a regulatory role in the entry into mitosis. However, tyrosine

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¹ The abbreviations used are: SFKs, Src family protein-tyrosine kinases; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; HA, hemagglutinin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; G₄₄, ganglioside G₄₄.
phosphorylation signaling thus far has not been fully characterized in cytokinesis.

In this study, we explored SFK-mediated tyrosine phosphorylation in cytokinesis by immunofluorescence staining for phosphotyrosine and live cell imaging experiments using two chemical compounds, PP2 and SU6656, and two membrane-anchored Csk mutants, all of which act as potent inhibitors of SFKs. We show that SFK-mediated tyrosine phosphorylation signaling that is spatially and temporally transmitted is required for completion of abscission in cytokinesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—cDNA encoding human c-Src (1–536; with 1 designating the initiator methionine) (provided by D. J. Fujita; Ref. 24) and Src-GFP (1–532; green fluorescent protein-fused, constitutively kinase-active) were described (25). Src258-GFP (1–258; kinase domain-deleted) was generated from Src-GFP. cDNA encoding human Lyn was provided by T. Yamamoto (26). GFP- and HA epitope-tagged Lyn constructs lacking the C-terminal negative-regulatory tail, Lyn-GFP (1–506; constitutively kinase-active), Lyn-HA (1–506; constitutively kinase-active), and Lyn-K275A-HA (1–506; kinase-inactive), were previously described (17). Rat Csk cDNA was provided by M. Okada and S. Nada (13). Src16-Csk and Lyn25-Csk were constructed by fusion with the respective N-terminal sequences of c-Src-(1–16) and Lyn-(1–25) at the N terminus of full-length Csk. cDNAs encoding human c-Abl-1b (27) and human Syk (28) were designed by P. Ferguson (29).

**Chemicals**—PP2 (30) and SU6656 (31) were purchased from Calbiochem. U0126 (Calbiochem), SB203580 (Sigma), and SP600125 (Biomol) were gifts from T. Murayama (32). All chemicals were dissolved in methanol/5328 bovine serum were blocked at S phase by addition of 1.6 μg/ml aphidicolin and nocodazole blocks, because prolonged treatment with nocodazole for mitotic arrest has adverse effects on synchronous release of cells from nocodazole arrest. HeLa S3 cells grown as monolayers in Isocove’s modified Dulbecco’s modified essential medium containing 5% fetal bovine serum. Transient transfection was performed using TransIT transfection reagent (Mirus), according to the manufacturer’s instructions, as reported previously (17, 34, 35).

**Immunofluorescence**—Immunofluorescence staining was detected using a Fluoview FV500 confocal laser scanning microscope with a 40 × 1.00 NA oil objective (Olympus) as described (17, 34–38). Cells were fixed in phosphate-buffered saline (PBS) containing 3% paraformaldehyde for 20 min, and permeabilized in PBS containing 0.1% saponin and 3% bovine serum albumin at room temperature. Cells were subsequently stained with an appropriate primary antibody for 1 h, washed with PBS containing 0.1% saponin, and stained with fluorescein isothiocyanate- or TRITC-conjugated secondary antibody for 1 h. For anti-Tyr(P) (4G10) staining of the midbody, cells were in situ treated with 0.5% Triton X-100 at 4 °C for 3 min before fixation with 3% paraformaldehyde (39). DNA was stained with 100 μg/ml propidium iodide for 20 min after treatment with 200 μg/ml RNase A (34). Emission signals were detected between 505 and 530 nm for fluorescein, at more than 580 nm for rhodamine and more than 650 nm for propidium iodide. Care was taken to ensure that there was no bleed-through from the fluorescein signal into the red channel (38).

**Time Lapse Phase-contrast Imaging**—HeLa cells cultured in Isocove’s modified Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum and 20 mM HEPES (pH 7.4) were placed on a 40 °C preheated stage of an inverted Zeiss Axiovert S100 deconvolution microscope with 10 × 0.30 N.A. and 20 × 0.50 N.A. objectives, and monitored in the presence of various inhibitors. Time lapse monitoring started within 5 min after addition of an inhibitor to living HeLa cells at each stage of mitosis. Each stage is morphologically classified as follows: metaphase (sister chromatids are aligned in the center of cell), anaphase (sister chromatids are separated and moved to opposite poles), telophase (the cleavage furrow is formed between the segregated chromosomes), and cytokinesis (two daughter cells are attached and connected by an intercellular bridge containing the midbody). Images were analyzed with MetaMorph version 4.5 (Universal Imaging).

**Cell Synchronization**—HeLa S3 cells were synchronized using successive aphidicolin and nocodazole blocks, because prolonged treatment with nocodazole for mitotic arrest has adverse effects on synchronous release of cells from nocodazole arrest. HeLa S3 cells grown as monolayers in Isocove’s modified Dulbecco’s modified essential medium containing 5% fetal bovine serum were blocked at S phase by addition of 1.6 μg/ml aphidicolin. After 16–20 h, the monolayers were washed twice with PBS. The cells were cultured for an additional 6–7 h, then exposed to 40 ng/ml nocodazole for 5–6 h. Round-shaped cells in mitotic stages were gently pipetted and collected by brief centrifugation. Mitotic cells were released from nocodazole treatment by extensively washing with pre-warmed PBS, and subsequently incubating in normal medium at 37 °C in suspension culture. Progression through mitosis was monitored every 10 min by immunofluorescence using anti-α-tubulin antibody and propidium iodide (see Fig. 3, A–C). For immunofluorescence, cells were directly fixed with 2% paraformaldehyde and then attached on coverslips by brief cytocentrifugation.
Western Blotting and Immunoprecipitation—Western blotting and immunoprecipitation were performed as described (17, 35, 39–42). In brief, cell lysates were prepared in Triton X-100 lysis buffer (50 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 4 mM EDTA, 100 mM NaF, and 1 mM Na$_3$VO$_4$) or RIPA lysis buffer (50 mM HEPES, pH 7.4, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 4 mM EDTA, 100 mM NaF, and 1 mM Na$_3$VO$_4$) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 50 µg/ml aprotinin, 100 µM leupeptin, and 25 µM pepstatin A) at 4 °C. The immune complexes were analyzed by SDS-PAGE and Western blotting, as described above.

RESULTS

Src Kinase Activity at the Early Phase of Mitosis Is Required for Abscission—There is emerging evidence that Src-mediated tyrosine phosphorylation signaling is involved in cell division, especially at the G$_2$-M transition, cleavage furrow progression, and completion of cytokinesis (18, 43, 44). To investigate the involvement of Src kinase activity in cytokinesis, living HeLa cells were monitored in a time lapse manner in the presence of PP2, a potent SFK inhibitor, under a deconvolution microscope. We observed that prolonged treatment with PP2 prevented cells from entering into M phase (Fig. 1A), consistent with previous reports that the G$_2$-M transition is blocked by inhibition of SFKs (18, 19). As shown in Fig. 1B, control cells completed cytokinesis within 144 ± 35 min after anaphase onset (n = 11, mean ± S.D.). Intriguingly, most cells that escaped from PP2-induced inhibition of G$_2$/M progression formed the cleavage furrow with a normal time course, but remained connected by an unusually elongated intercellular bridge (Fig. 1C). These results indicate that a defect in cytokinesis takes place during the terminal step in cytokinesis called abscission.

To examine whether the involvement of Src kinase activity in abscission was temporally controlled, living HeLa cells treated with PP2 from a particular stage of M phase were monitored in a time lapse manner under a deconvolution microscope. When PP2 was added to cells before cleavage furrow formation, most cells underwent progression of the cleavage furrow, but remained connected in a pair by an unusually elongated intercellular bridge (Fig. 2, A and C, filled bars, before metaphase and anaphase). Additionally, cells sometimes appeared to exhibit a defect in cleavage furrow progression (Fig. 2C, shaded bars, before metaphase and anaphase/anaphase), in agree-
PP2 treatment

A

anaphase

0 min 20 min 50 min 110 min 510 min

B
telophase

0 min 30 min 60 min 100 min 130 min

C

DMSO

PP2

SU6656

n = 66

n = 17

n = 14

n = 8

n = 13

n = 17

before metaphase

before metaphase

metaphase

anaphase

telophase

cytokinesis

metaphase

telophase

Cells (%)

0 50 100

0 50 100

FIGURE 2. Requirement of SFK activity for cytokinesis. HeLa cells were treated with 10 μM PP2 or 2 μM SU6656 at each stage of the cell cycle, and then monitored at 10-min intervals. A and B, time lapse phase-contrast images of HeLa cells treated with PP2 at the onset of anaphase (A) or telophase (B). A cell treated with PP2 at the onset of anaphase (0 min) formed the cleavage furrow (20 min), but remained connected by an elongated intercellular bridge at 510 min (A). However, a cell treated with PP2 at the onset of telophase (0 min) was separated into two daughter cells within 130 min (B). Note that no discernible difference was observed in cell morphology and motility between cells treated with PP2 at the onset of anaphase (A) and those at telophase (B). Intercellular bridges containing the midbody were observed at the center of circles. Arrows indicate the two separating daughter cells. Scale bars, 10 μm. C, HeLa cells were treated with 0.1% DMSO (DMSO) (solvent control), 10 μM PP2, or 2 μM SU6656 from the indicated stages of the cell cycle, and monitored at 10-min intervals. Cells were classified as follows: an intercellular bridge was cleaved within 3 h (normal, open bars) or connected for more than 3 h (abscission failure, filled bars) or others (shaded bars) such as arrest of metaphase progression.

PP2 Treatment Induces Abscission Failure in an Adhesion-independent Manner—SFKs play a role in cell adhesion and migration by controlling cytoskeletal reorganization (11). However, when cells were treated with PP2 after cleavage furrow formation, abscission was completed with a normal time course (Fig. 2, B and C, open bars, telophase and cytokinesis). Furthermore, treatment of cells with SU6656 (another selective SFK inhibitor; Ref. 31) in metaphase/anaphase induced abscission failure similar to that induced by PP2 (Fig. 2C). These results suggest that before cleavage furrow formation, SFK catalytic activity is required for abscission.

PP2 Treatment Decreases Tyrosine Phosphorylation Levels at the Midbody—To scrutinize tyrosine phosphorylation signaling specific for M phase, we examined the localization of tyrosine-phosphorylated proteins by in situ detergent extraction and confocal fluorescence microscopy. Immunostaining of Triton X-100-treated cells with anti-phosphotyrosine (Tyr(P)) antibody revealed the localization of tyrosine-phosphorylated proteins to the midbody (Fig. 4A). Because the tyrosine-phosphorylated proteins found at the midbody were in particular resistant to Triton X-100 extraction, they may be tightly associated with cytoskeletal components at the midbody. The levels of tyrosine phosphorylation were higher at the plus-ends (+) of microtubules of the midbody than at the minus-ends (−), and a faint signal was detected at the midbody matrix (Fig. 4A).

To investigate whether tyrosine phosphorylation signaling at the midbody was involved in PP2-induced abscission failure, we quantitated the fluorescence intensity of anti-Tyr(P) antibody...
reacted to the midbody region in PP2-treated cells. 12-h treatment of HeLa cells with PP2 induced a phenotype of cells having an unusually elongated intercellular bridge, as observed in time lapse monitoring of PP2-treated cells (Figs. 1 and 2), and significantly reduced the levels of tyrosine phosphorylation at the midbody (Fig. 4, B and C). These results suggest that PP2-induced abscission failure may be caused by reduced levels of tyrosine phosphorylation at the midbody.

In contrast, a 30-min treatment of HeLa cells with PP2 had no effect on increased levels of tyrosine phosphorylation at the midbody and the appearance of a normal, short intercellular bridge (Fig. 4C; data not shown), despite a profound suppression of the kinase activity of c-Src (Fig. 4D). Once the midbody is formed, tyrosine phosphorylation signaling at the midbody may be insensitive to PP2 treatment. In other words, PP2 treatment from metaphase to anaphase, but not after cleavage furrow formation, is critical for induction of abscission failure (Figs. 2 and 3, A–F). Taken together, we suggest that abscission is dependent on tyrosine phosphorylation signaling at the midbody, which is regulated by SFK kinase activity at the early phase of mitosis.

Tyrosine Phosphorylation Signaling Is Transmitted to the Midbody via Recycling Endosomes—Abscission is dependent on vesicle transport from intracellular organelles to the cleavage furrow and the midbody during late mitosis (2–10). It was noted that both Src-GFP and Lyn-GFP were barely seen at the midbody in Triton X-100-extracted HeLa cells (Fig. 4E) where tyrosine phosphorylation was clearly retained at the midbody (Fig. 4, A and B). We thus hypothesized that proteins may be tyrosine-phosphorylated downstream of SFKs at the other locations except for the midbody in the early phase of mitosis, and then delivered to the midbody by vesicle transport.

Rab11, a small GTPase, localizes preferentially to the recycling endosome and is required for proper recycling of endosome organization and the recycling of vesicles to the plasma membrane (46). Expression of the dominant-negative Rab11S25N mutant that can disrupt the function of recycling endosomes (29) inhibited cytokinesis and prominently induced either a phenotype with an elongated intercellular bridge or a binuclear phenotype (Fig. 5). Expression of Rab11S25N was found to decrease the levels of tyrosine phosphorylation at the midbody, without affecting SFK kinase activity (Fig. 5E). These results suggest that tyrosine-phosphorylated proteins are delivered to the midbody through Rab11-driven recycling endosomes.

**FIGURE 3.** Cell adhesion-independent SFK signaling in abscission. M phase-arrested HeLa S3 cells were released from nocodazole treatment by washing with PBS, and then incubated at 37°C in suspension culture for the indicated times in the presence or absence of 10 μM PP2 (see details under “Experimental Procedures”). A–C, progression through M phase was examined every 10 min by immunofluorescence using propidium iodide (DNA) and anti-α-tubulin antibody. A and B, cells entering in M phase were classified as indicated. C, representative images of cells at 40 and 90 min from release are shown. Scale bars, 20 μm. D, PP2 treatment of synchronous HeLa S3 cells in suspension culture. Cells were treated with 0.1% MeSO (a) or 10 μM PP2 (b and c) after a 10-min (a and b) or 50-min (c) recovery from nocodazole arrest. Cells were fixed after 90 or 180 min for immunostaining. E and F, cells exhibiting the midbody or binuclei were quantitated after 90 or 180 min (F) recovery from nocodazole arrest. Data represent mean (±S.D.) from one or three independent experiments. *, p < 0.05; **, p < 0.01, Student’s t test). DMSO, dimethyl sulfoxide.
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Panel A: Nomarski images of cells with different treatments. Images show the localization of actin filaments (α-tubulin) and phosphorylated tyrosine (pTyr) with and without TX100 treatment.

Panel B: Control and PP2-treated cells showing the distribution of pTyr under + TX100 conditions.

Panel C: Graph showing the integrated intensity of pTyr in the midbody region under different conditions. The data indicates statistical significance (p < 0.05) for the control and 30 min PP2 treatment groups.

Panel D: Western blot (WB) analysis comparing the expression of Src in control and PP2-treated samples. The blot shows the reduction in Src expression with PP2 treatment.

Panel E: Images of cells expressing Src-GFP and Lyn-GFP with and without TX100 treatment.
Cytokinesis Failure Is Induced by Csk-mediated Inhibition of SFKs—

The activation of c-Src at mitosis is controlled by changes in the phosphorylation state of the C-terminal negative-regulatory tyrosine residue of SFKs by Csk and the transmembrane protein-tyrosine phosphatase α in membrane fractions (15, 20–23). To scrutinize the involvement of SFKs in abscission, we constructed two membrane-anchored Csk chimeras, Src16-Csk and Lyn25-Csk (Fig. 6A). Both were predominantly localized to the plasma membrane through the N-terminal acylation signals of c-Src and Lyn, whereas wild-type Csk was mostly distributed in the cytosol (Fig. 6, B–D). Expression of Src16-Csk and Lyn25-Csk efficiently decreased the activity of autophosphorylation of endogenous SFKs compared with that of Csk (Fig. 6E), consistent with previous reports that membrane-anchored Csk chimeras, which possess myristoylation or isoprenylation signals, potently suppress SFK activity (14–16).

When HeLa cells transfected with Src16-Csk or Lyn25-Csk were cultured for 24 h, the levels of tyrosine phosphorylation at the midbody were markedly suppressed (Fig. 6, F and G), and the number of cells with an unusually elongated intercellular bridge containing the midbody increased appreciably (Fig. 6). These phenotypes were similar to those observed in cytokinesis failure induced by PP2. In the midbody region, proteins required for abscission are tyrosine-phosphorylated downstream of SFKs at the metaphase to anaphase transition, and subsequently transported to the midbody by vesicle transport during telophase and cytokinesis.
larly observed in A431 and COS-1 cells transfected with membrane-anchored Csk (data not shown) and in some HeLa cells overexpressing wild-type Csk (47). Furthermore, Lyn25-Csk was more potent in cytokinesis failure than Src16-Csk (Fig. 7A), in accordance with the finding that Lyn25-Csk induced stronger inhibition of endogenous c-Src and Lyn kinase activities than Src16-Csk (Fig. 6E). Importantly, it was evident that cells were fully rescued from Src16-Csk- or Lyn25-Csk-induced inhibition of cytokinesis by co-expression of constitutively kinase-active SFK (Src-GFP, Lyn-GFP, or Lyn-HA), but not kinase-inactive SFK (Src258-GFP or Lyn-K275A-HA) (Figs. 6A and 7). However, co-expression of the irrelevant non-receptor-type tyrosine kinase c-Abl or Syk, both of which are kinase-active, did not overcome Src16-Csk- or Lyn25-Csk-induced inhibition of cytokinesis (47).

**FIGURE 6.** Inhibition of tyrosine phosphorylation at the midbody by expression of membrane-anchored Csk mutants. A, schematic representations of the constructs used in this study. The Src homology (SH) domains, the kinase domain, the negative regulatory tyrosine residue (Regulatory-Tyr), green fluorescent protein (GFP), and the HA tag are shown. Src16, the N-terminal c-Src sequence (16 amino acid residues); Lyn25, the N-terminal Lyn sequence (25 amino acid residues); Lyn-K275A, kinase-inactive Lyn mutant. B–D, COS-1 cells transiently expressing Csk (B), Src16-Csk (C), and Lyn25-Csk (D) were doubly visualized with anti-Csk antibody and propidium iodide (DNA). Scale bars, 10 μm. E, HeLa cells transiently transfected with the indicated constructs were cultured for 24 h. Equal amounts of Triton X-100 cell lysates (input lysates) were analyzed by Western blotting with anti-Csk and anti-actin (loading control) antibodies (bottom panel). c-Src or Lyn immunoprecipitates from equal amounts of Triton X-100 cell lysates were analyzed by Western blotting with anti-Src[pY418], anti-Src, and anti-Lyn antibodies. Anti-Src[pY418] antibody recognizes both active forms of c-Src and Lyn. IP, immunoprecipitation; WB, Western blotting. F, HeLa cells transiently transfected with none (Control), Src16-Csk, or Lyn25-Csk were cultured for 24 h. After Triton X-100 treatment, cells were visualized by Nomarski optics and anti-Tyr(P) and anti-Csk immunofluorescence. Insets show a magnification of the areas indicated by dotted squares. Arrows indicate the midbody matrix. Scale bars, 10 μm. G, integrated fluorescence intensity of anti-Tyr(P) antibody reacted to the midbody region (+, p < 0.01, Student’s t test). The plot represents each individual value of the integrated intensity in the midbody region (open circle).
inhibition of cytokinesis (Fig. 7), in agreement with previous work that the Abl inhibitor Gleevec had no effect on cytokinesis, mitosis, or formation of cholesterol- or GM1-rich rings (43). These results indicate the requirement of the substrate specificity of SFKs. In addition, no effect of Lyn25-GFP alone on cytokinesis ruled out the possibility that expression of any acylated proteins brings about cytokinesis failure (Figs. 6A and 7A). Thus, these results suggest that the defect in abscission is primarily caused by inhibition of the kinase activities of endogenous SFKs.

**SFK-mediated ERK Activation at the Midbody Is Required for Abscission**—Because SFKs were not associated with the midbody (Fig. 4E), we next searched for a protein(s) that is tyrosine-phosphorylated and associated with the midbody. ERK1/2 (p44/p42 MAP kinases) were found to locate at the midbody (Fig. 8A), in agreement with previous reports (48–50). Furthermore, ERK1/2 were delivered to the midbody via Rab11-driven vesicle transport (Fig. 8C) and remained strongly associated with the midbody even after Triton X-100 treatment (Fig. 8B).

Next, to ask whether ERK1/2 activation was required for abscission, we treated HeLa cells with U0126, a MEK1/2 inhibitor. Treatment with U0126 in metaphase/anaphase induced abscission failure and retardation of progression from metaphase to cytokinesis (Fig. 8D). This retardation was probably due to the MEK-ERK pathway in regulating mitotic spindles and kinetochores (48–52). We then treated HeLa cells with U0126 during cytokinesis. As shown in Fig. 8, E and F, abscission was obviously prevented by treatment with U0126, but not SB203580, a p38 MAP kinase inhibitor, and SP600125, a c-Jun NH₂-terminal kinase (JNK) inhibitor. Consistent with U0126-induced abscission failure (Fig. 8, D–F), brief treatment with U0126 strikingly reduced tyrosine phosphorylation levels at the midbody (Fig. 8, G and H). These results suggest that ERK1/2 activation is critical for abscission. We thus assumed that ERK1/2 are tyrosine-phosphorylated proteins downstream of SFKs.

Given that ERK1/2, which are activated through MEK1/2-catalyzed dual phosphorylation of threonine and tyrosine residues in the TEY sequence, are known to regulate multiple functions during mitosis (48–54), we examined the activities of ERK1/2 in cytokinesis in the presence or absence of PP2 using anti-pERK1/2 antibody. Because Triton X100-insoluble ERK1/2 were principally localized at the midbody during cytokinesis in HeLa cells (Fig. 8B), we used HeLa S3 cells that can be highly synchronized in cytokinesis (see Fig. 3, A–E) and immunoprecipitated ERK1/2 from RIPA lysates of Triton X-100-insoluble HeLa S3 cells. When synchronized HeLa S3 cells were treated with PP2 before furrow formation, levels of dual phosphorylated ERK1/2 were greatly decreased in cytokinesis (Fig. 8I, middle panel, lane b). However, treatment with PP2 after furrow formation marginally affected levels of dual phosphorylated ERK1/2 (middle panel, lane c). In addition, levels of total ERK1/2 were not affected by PP2 treatment (left panel, lanes a–c). Furthermore, similar to anti-pERK1/2 antibody, anti-Tyr(P) antibody recognized dual phosphorylated ERK1/2 (Fig. 8I, right panel). Thus,
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D U0126 treatment from metaphase/anaphase

E U0126 treatment in cytokinesis

G + TX100

H Integrated intensity of pTyr in the midbody region

I Synchronized cells in cytokinesis

WB: Anti-ERK2

IP: Anti-ERK2
the midbody-specific tyrosine-phosphorylated proteins were identified as dual phosphorylated ERK1/2. Taken together, these results suggest that SFK activities during early mitosis mediate ERK activation at the midbody through the Rab11-driven vesicle transport, leading to completion of abscission.

DISCUSSION

In the present study, we show that SFK-mediated tyrosine phosphorylation signaling at the midbody is required for abscission in HeLa cells. Indeed, tyrosine-phosphorylated proteins, resistant to Triton X-100 extraction, are clearly visualized at the midbody by anti-Tyr(P) immunofluorescence. Although there are barely detectable protein levels of SFKs at the midbody, SFK-mediated tyrosine phosphorylation signaling at the early phase of mitosis is delivered to the midbody by Rab11-driven vesicle transport. Inhibition of SFKs or MEK1/2 in M phase decreases levels of ERK1/2 tyrosine phosphorylation at the midbody and causes abscission failure, accompanied by formation of an unusually elongated intercellular bridge. Recruitment of activated ERK1/2, which are phosphorylated by MEK1/2 downstream of SFKs, to the midbody plays an important role in completion of abscission.

Previously, it was found that SFKs, such as c-Src, c-Yes, and Fyn, are transiently activated at the transition from G2 phase to mitosis in the cell cycle and required for entry into mitosis before nuclear envelope breakdown (18, 19). The equatorial membrane domain enriched in ganglioside G MOV, cholesterol, and SFKs are formed at the contractile ring in late anaphase, and activation of Src is required for cleavage furrow progression (43). Active SFKs that are linked to Diaphanous-related formins are reported to participate in completion of cytokinesis, and cells injected with anti-Src antibody during mitosis become binucleate (44).

Our findings indicate that the tyrosine-phosphorylated proteins present at the midbody are identified as activated ERK1/2, which are phosphorylated on threonine and tyrosine residues in the TEY sequence, due to the following similarities between midbody-specific tyrosine-phosphorylated proteins and activated ERK1/2: (i) they are detected with anti-Tyr(P) antibody (Figs. 4A and 8F), (ii) both are strongly associated with the midbody after Triton X-100 treatment (Figs. 4A and 8B), (iii) they are delivered to the midbody via Rab11-mediated vesicle transport (Figs. 5 and 8C), (iv) their tyrosine phosphorylation is dependent on SFK kinase activity in early mitosis (Figs. 4, B and C, and 8l), and (v) brief treatment with U0126 inhibits midbody-specific tyrosine phosphorylation and abscission (Fig. 8, G and H; data not shown). Taken together, ERK1/2 activation at the midbody is required for abscission.

SFKs were reported to localize to the mitotic spindles (55) and midbody (43). However, it is unlikely that SFK-mediated ERK1/2 activation takes place at the midbody, because ERK1/2 recruitment and tyrosine phosphorylation at the midbody are attenuated by disruption of Rab11-driven vesicle transport (Figs. 5, C and D, and 8C) and the kinase activity of SFKs between metaphase and anaphase (before the midbody is formed) is required for abscission (Figs. 2 and 3). SFK-mediated tyrosine phosphorylation and ERK1/2 activation are required for completion of abscission in a time-dependent manner (Figs. 3F and 8F). In addition, Src-GFP and Lyn-GFP were barely detectable at the midbody after Triton X-100 treatment in HeLa cells (Fig. 4E), although this is inconsistent with previous work that active SFKs were visualized at the midbody by immunofluorescence using an antibody reactive to the C-terminal, nonphosphorylated negative-regulatory tyrosine residues of SFKs (43).

We can illustrate a model for three sequential waves of SFK-mediated tyrosine phosphorylation during M phase (Fig. 9A). First, the kinase activity of SFKs is required for the G2–M transition (step a). Second, activation of SFKs is required for cleavage furrow progression (step b). Third, activated ERK1/2, which is phosphorylated by MEK1/2 through tyrosine phosphorylation signaling of SFKs, delivered to the midbody is required for completion of abscission (step c). Furthermore, as illustrated in Fig. 9B, ERK1/2 are activated by Src-mediated signaling at the plasma membrane during metaphase and/or anaphase, and is conveyed to the Rab11-associated recycling endosome by internalization. Subsequently, ERK1/2 are in turn delivered to the midbody by vesicle transport during telophase and/or cytokinesis. Alternatively, ERK1/2 activation mediated by SFKs may take place on recycling endosomes, because c-Src is present on Rab11-associated endosomes (Ref. 56; data not shown). In either case, it should be emphasized that delivery of ERK1/2 to the midbody is important for abscission.

However, the mechanism for regulation of abscission via MEK-ERK signaling is unknown, although ERK1/2 are associated with the midbody (Fig. 8, A–C) (48–50). MEK and ERK are well known to be present on the mitotic spindles and regulate the assembly and maintenance of mitotic spindles in metaphase and anaphase (48–52). There is an increasing number of similarities in the components and function of the midbody in animals and the phragmoplast, which plays an essential role during...
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FIGURE 9. Model of the sequential action of SFKs in M phase. A, the catalytic activity of SFKs is required for mitosis at three different sequential steps: the G2-M transition (arrow a; Ref. 18), cleavage furrow progression (arrow b; Refs. 43 and 44), and abscission (arrow c; this study). DNA and microtubules are shown. The bundled microtubules observed in cytokinesis indicate the midbody, b, during the course of metaphase and anaphase, ERK1/2 are phosphorylated and activated by MEK downstream of SFK signaling at the plasma membrane and/or Rab11-associated recycling endosomes (Rab11(+) endosomes). Activated ERK1/2 possibly together with activated MEK are subsequently delivered to the midbody via vesicle transport. The dominant-negative Rab11S25N prevents vesicle transport of activated ERK1/2 from recycling endosomes to the midbody. Thus, the recruitment of activated ERK1/2, which are phosphorylated by MEK downstream of SFKs, to the midbody is implicated to play a role in abscission.

cytokinesis in plants (2). Recent studies showed that the tobacco MAPK cascade positively regulates the phragmoplast in plants by phosphorylation of NtMAP65-1, a microtubule-associated protein, that leads to destabilization and turnover of the phragmoplast (53, 54). Inhibition of SFKs or MEK1/2 induces an unusually elongated intercellular bridge containing the midbody (Figs. 1C, 2A, 3F, 4B, 6F, 7, and 8D). This means that the midbody may be stabilized by blockade of the SFK-mediated MEK-ERK pathway. Thus, regulation of microtubule-associated proteins could be involved in completion of abscission far downstream of midbody-specific ERK signaling. It is now of interest to dissect how activated ERK1/2, present at the midbody, can regulate abscission in cytokinesis.

Despite accumulating evidence for the involvement of SFKs in cell cycle progression (11, 18, 19, 42, 43), to further ascertain the involvement of SFKs in abscission, we used a fibroblastic cell line transformed by the SV40 large T antigen, derived from mice lacking c-Src, c-Yes, and Fyn (SYF cells; Ref. 33). It was previously reported that SYF cells can normally proliferate (33). We found that inhibition of MEK-ERK signaling did not affect abscission in SYF cells, consistent with very limited tyrosine phosphorylation signals detected by anti-Tyr(P) immunofluorescence at the midbody in untreated SYF cells (data not shown). In addition, we found that in some cell types, such as human breast cancer MCF-7 cells and human colon cancer HCT116 cells, abscission failure was not induced by mitosis-specific SFK or MEK/ERK inhibition (data not shown). What is the difference in completion of abscission between SFK/MEK/ERK-dependent and -independent manners? Abscission in SYF, MCF-7, and HCT116 cells might involve the following common features that we observed: (i) barely detectable levels of tyrosine phosphorylation at the midbody, (ii) completion of abscission at very rapid rates, and (iii) formation of a very short intercellular bridge containing the midbody. Assumably, abscission may be regulated by more than one mechanism including SFK/MEK/ERK-mediated signaling. Detailed studies will be required for elucidating an SFK/MEK/ERK-independent mechanism.

On the basis of these results, we identify midbody-associated ERKs downstream of SFKs in SFK-dependent cytokinesis in HeLa cells and demonstrate that the precise temporal and spatial regulation of this signaling is important for abscission. Further studies of regulation and function of SFK/MEK/ERK will contribute to a better understanding of the regulatory circuits that control abscission.

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