Mps1 is a protein kinase that regulates normal mitotic progression and the spindle checkpoint in response to spindle damage. The levels of Mps1 are relatively low in cells during interphase but elevated in mitosis or upon activation of the spindle checkpoint, although the dynamic range of Mps1 expression and the Mps1 catalytic mechanism have not been carefully characterized. Our recent structural studies of the Mps1 kinase domain revealed that the carboxyl-terminal tail region of Mps1 is unstructured, raising the question of whether this region has any functional role in Mps1 catalysis. Here we first determined the cellular abundance of Mps1 during cell cycle progression and found that Mps1 levels vary between 60,000 per cell in early G2 and 110,000 per cell during mitosis. We studied phosphorylation of a number of Mps1 substrates in vitro and in culture cells. Unexpectedly, we found that the unstructured carboxyl-terminal region of Mps1 plays an essential role in substrate recruitment. Kinetics studies using the purified recombinant wild type and mutant kinases indicate that the carboxyl-terminal tail is largely dispensable for autophosphorylation of Mps1 but critical for trans-phosphorylation of substrates in vitro and in cultured cells. Mps1 mutant without the unstructured tail region is defective in mediating spindle assembly checkpoint activation. Our results underscore the importance of the unstructured tail region of Mps1 in kinase activation.

Spindle checkpoint events are critical for faithful cell duplication and organism development. Aberrations in checkpoint control mechanisms often have serious consequences and can potentially lead to cancer (1). The mitotic spindle checkpoint is frequently altered in cancer cells and facilitates mitotic progression. Consequently, it fails to prevent chromosome mis-segregation and can give rise to aneuploid cancer cells (1, 2). Although it is known that mitotic progression is controlled by a number of protein kinases, including the series of cyclin-dependent kinases (Cdks), the precise molecular mechanisms by which checkpoint signaling pathways modulate cyclin-dependent kinases to halt cell cycle progression remains incompletely understood (3).

Mps1/TTK is one of the major kinases that helps regulate mitotic progression and mitotic spindle checkpoint signaling (4). Originally identified as a protein kinase that is overexpressed in human tumor cells and a kinase associated with cell proliferation in mouse embryonic carcinoma cells (5–7), Mps1/TTK functions in several aspects of cell cycle control including spindle pole body duplication (8), mitotic spindle checkpoint (9–13), proper mitotic progression (14), centrosome duplication (15), chromosome alignment (16), and cytokinesis (14). Alterations in Mps1/TTK expression and its kinase activity cause meiotic errors (17, 18), aneuploidy, and developmental defects (19, 20). The findings that Mps1/TTK alterations are associated with human cancer and that cells require Mps1/TTK for survival and proliferation raised the possibility that Mps1/TTK can be exploited as a novel therapeutic target for cancer treatment (21, 22).

The success of this novel therapeutic approach requires a detailed understanding of its enzymatic mechanisms and cellular abundance. Currently the kinetic mechanism for Mps1/TTK kinase has yet to be characterized. Here we measured the cellular abundance of Mps1/TTK using quantitative immunoblotting during cell cycle progression and conduct a kinetics analysis of Mps1/TTK phosphorylation. We found that the abundance of Mps1/TTK is approximately 30–40-fold lower than Cdk1 depending the stage of cell cycle. To understand the catalytic mechanism of Mps1/TTK, we investigated Mps1/TTK substrate phosphorylation in vitro and in cultured cells. We found that the unstructured carboxyl-terminal tail of Mps1/TTK is largely dispensable for autophosphorylation but necessary for optimal substrate phosphorylation. These results suggest that it might be possible to develop allosteric small molecule inhibitors that target the carboxyl-terminal region of Mps1/TTK to perturb its function in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture and Antibodies—HeLa S3 and SW480 cells, purchased from American Type Culture Collection (Rock-
ville, MD), were maintained in DMEM media (Invitrogen) with 10% FBS (Invitrogen) and 100 units/ml penicillin plus 100 μg/ml streptomycin (Invitrogen). Insect Hi-Five cells were grown at 27 °C in Insect Express media (Lonza). Antibodies against Mps1/TTK (SC-19, Cdk1 (SC-54), and cyclin B1 (SC-245) were purchased from Santa Cruz Biotechnology. FLAG antibody was purchased from Agilent Technology. Mps1 phospho-Thr-686 antibody has been described previously (23). Rabbit Mps1/TTK antibody against the amino-terminal domain of Mps1/TTK was raised using purified recombinant TTK/Mps1-NTD (1–270), prepared from *Escherichia coli* as described previously (23). The antibody was produced by QED Biosciences (San Diego) and affinity-purified using an antibody purification kit (Bio-Rad).

**Expression Vectors**—Mammalian Mps1 and Smad2 expression vectors have been described previously (24, 25). To produce wild type and a carboxyl-terminal tail deletion mutant of Mps1, full-length and Mps1 1–792 were PCR-amplified and inserted into pFast-Bac-GST-3C-thrombin-His-TEV-FLAG vector, a derivative of pFast (Invitrogen), such that the amino terminus of Mps1 is fused to GST followed by a 3C protease cleavage site, and the carboxyl terminus of Mps1 is fused in-frame to a thrombin cleavage site followed by a His$_{6}$ tag and a FLAG tag. Bacmid preparation and transfection of sf9 cells were performed as described by the manufacturer (Invitrogen). Single plaques were picked and amplified to obtain high titer viral stocks used for subsequent infection.

Recombinant Mps1 protein was produced by infecting 2 liters of Hi-Five cells grown to a density of 2 × 10$^{6}$ cells/ml with the specified recombinant baculovirus at a multiplicity of infection of 1. Forty-eight hours later the infected cells were harvested by centrifugation of the cell suspension for 10 min at 3000 rpm. The cell pellets were weighed and flash-frozen in liquid nitrogen, and stored at −80 °C until needed.

To express the amino-terminal domain of Mps1 (1–270), a pair of primers with BamHI and XhoI sites attached were used to PCR-amplify the desirable fragment of Mps1 and inserted into pET28b-His$_{6}$-SUMO vector (26). The expression vector was used to transform *E. coli* BL21(DE3) for recombinant protein production. GST-borealin was a generous gift from Dr. Kops, and recombinant GST-borealin was purified as described previously (16).

Transient expression of Mps1 and Mps1 mutants was achieved by subcloning of wild type and mutant cDNA into the pRK5-myc expression vector. Stable expression of siRNA-insensitive variants of Mps1 using retroviral-mediated gene transfer and RNAi has been described previously (25).

**Purification of Mps1-NTD**—To purify Mps1-NTD, bacteria with pET28b-His$_{6}$-SUMO-Mps1-NTD were grown at 37 °C to a density of 0.7 (A$_{600}$) before adding 0.1 mM isopropyl 1-thio-β-D-galactopyranoside to induce protein expression. The cells were harvested after further incubation for 4 h at 28 °C and sonicated in buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole). After centrifugation, the supernatant was incubated with Ni-NTA-agarose (Qiagen) at 4 °C. His fusion proteins bound on beads were washed extensively with washing buffer B (identical to buffer A except 20 mM imidazole) 3 times and eluted with buffer C (identical to buffer A except 200 mM imidazole). The protein concentration was determined by the Bradford assay (Bio-Rad). Purified protein was frozen in liquid nitrogen and stored in individual aliquots at −80 °C.

**Protein Purification of Mps1 and Mps1ΔD**—For purification of recombinant Mps1 and Mps1ΔD from Hi-Five cells, cell pellets were disrupted by 3 freeze-thaw cycles in 80 ml of ice-cold NETN buffer (50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.2% Nonidet P-40, 1 mM DTT) per liter of cell suspension. After centrifugation at 10,000 rpm for 30 min, glutathione-Sepharose beads (GE Healthcare) washed with PBS were added to the supernatant, and the mixtures were incubated at 4 °C for 1 h. GST fusion proteins bound to beads were washed extensively with the NETN buffer followed by 10 volumes of 3C cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 1 mM DTT). Mps1 was eluted from the glutathione-Sepharose beads by incubating the beads with recombinant 3C protease according to the manufacturer’s (GE Healthcare) instructions. The 3C-eluted protein was subsequently applied onto an Ni-NTA-agarose (Qiagen) column. The column was washed with 5 column volumes of washing buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) twice following by 5 column volumes of PBS buffer and resuspended in the thrombin cleavage buffer. Recombinant thrombin (Sigma) was used to cleave the protein from the Ni-NTA beads. Eluates were further dialyzed against storage buffer (50 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.5 mM DTT, and 20% glycerol) to remove imidazole and stored for further use.

**Western Blot**—Purified proteins were resolved by 12% SDS-PAGE and electrophoretically transferred to nitrocellulose. Western blot analysis was performed using the indicated antibodies and corresponding HRP-conjugated secondary antibodies (GE Healthcare) using a West Dura ECL detection kit (Pierce).

**In Vitro Kinase Assay**—The kinase activity of Mps1 or Mps1 mutant was measured in 10 μl of 1× kinase buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM dithiothreitol, 10 mM 2-mercaptoethanol, 5% glycerol) supplemented with 50 mM MgCl$_{2}$, 100 μM ATP (Sigma), and 2 μCi of [γ-33P]ATP (3000 Ci/mmol, 9.25 MBq) (PerkinElmer Life Sciences). The desired amount of Mps1-NTD (50–200 nm) or MBP was used as the substrates of trans-phosphorylation. The enzyme and substrate were mixed and incubated for 1 h at 30 °C unless otherwise indicated. Reactions were quenched by the addition of 5 μl of 4× SDS loading buffer at various times (0–120 min) and then boiled for 5 min before SDS-PAGE. The gel was fixed with the destaining solution (10% acetic acid, 45% methanol, and 45% H$_{2}$O) and dried. The amount of 33P incorporation into the substrate and Mps1 itself was detected by autoradiography using a Typhoon PhosphorImager.

**Steady State Enzyme Kinetics Analysis**—Two-substrate analysis using the [γ-33P]ATP assay was performed by varying the ATP and Mps1-NTD concentrations from 30 to 540 μM and from 50 to 250 μM, respectively. Data were fit to the
Mps1 Abundance and Kinetics Analysis

Michaelis-Menten equation (Equation 1) to derive apparent \(V_{\text{max}}\) and \(K_m\) values for each substrate concentration using nonlinear least-squares fitting (MATLAB7.6 software). The complete set of velocity data were globally fit to expressions describing either a ternary complex (Equation 2) or a ping-pong enzymatic mechanism (Equation 3). The nonlinear, least-squares methods were used to ascertain the best fit parameters for each experiment. The equations for kinetics analysis were as described by Cleland (27).

For single substrate kinetics measurements, see Equation 1.

\[
V = \frac{V_{\text{max}}[S]}{(K_m + [S])}
\]

(Eq. 1)

For the discrimination between sequential and ping-pong mechanism of two-substrate kinetics, data were fit to Equations 2 and 3.

\[
V = \frac{V_{\text{max}}[ATP][S]}{[ATP][S] + [ATP]K_mS + [S]K_{m,ATP} + K_mK_{s,ATP}}
\]

(Eq. 2)

\[
V = \frac{V_{\text{max}}[ATP][S]}{[ATP][S] + [ATP]K_mS + [S]K_{m,ATP}}
\]

(Eq. 3)

In these equations, \(v\) represents the measured velocity, \(V_{\text{max}}\) is the maximum velocity; ATP and \(S\) are substrates; \(K_m,ATP\) and \(K_m,S\) are the Michaelis constants for ATP, and Mps1-NTD separately at saturating concentrations of the other substrate, \(K_{s,ATP}\) is the association constant of Mps1-NTD for free Mps1, and \(K_{s,ATP}\) is the dissociation constant of ATP for free Mps1. The two-site interaction factor \(\alpha\) is the ratio of the Michaelis constant to the dissociation constant factor, and it quantifies the degree to which the binding of one substrate either increases (<1) or decreases (>1) the affinity of the enzyme for the other substrate (Equation 4).

\[
\alpha = \frac{K_{m,S}}{K_{s,ATP}} = \frac{K_{m,ATP}}{K_{s,ATP}}
\]

(Eq. 4)

RESULTS

Cellular Abundance of Mps1, Cdk1, and Cyclin B1—It is known that the abundance of Mps1 is cell cycle-regulated (5, 12), yet little quantitative information is available for the absolute cellular abundance of Mps1, Cdk1, and cyclin B1 during cell cycle progression in mammalian cells. To determine the cellular abundances of these proteins, we first performed quantitative immunoblotting of HeLa cell lysates collected at different stages of the cell cycle (Fig. 1A). Recombinant Mps1, Cdk1, and GST-cyclin B1 were purified from either bacteria (Mps1-NTD) or insect cells (Cdk1 and cyclin B1), and the amounts of recombinant protein were determined by Coo massie Blue staining. Standard curves were generated by serial dilutions of known amounts of recombinant proteins followed by immunoblotting analysis (Fig. 1B). Shown in Table 1 is the abundance of Mps1, Cdk1, and cyclin B1 during cell cycle progression. In agreement with previous observations, the cyclin B1 levels show the most dynamic changes during cell cycle progression (Fig. 1B and Table 1). In G1 cells cyclin B1 is undetectable, contrasted by peaks of ~1.6 million molecules per cell in G2/M cells (Table 1). In contrast, Cdk1 levels are relatively constant throughout the cycle level and slightly elevated in G2/M cells with an estimated ~2.7 million molecules per cell. The abundance of Mps1 is much lower than cyclin B1 or Cdk1. Mps1 is very low in interphase cells (~57,000 molecules per cell) but is modestly increased in G2/M cells (~110,000 molecules per cell). The estimated increase in Mps1 abundance is consistent with the relative change in Mps1 levels quantified by mass spectrometry (28). Overall we estimate that Cdk1 is about 30-fold more abundant than Mps1 (Table 1). It is well established that Mps1 acts upstream in the checkpoint signaling cascade to prevent the decline of cyclin B1 and anaphase entry. Low abundance of Mps1 rela-
Table 1: Quantification of absolute Mps1, cyclin B, and Cdk1 cellular concentrations in HeLa cells

| Molecules per cell | S          | G2         | G2/M       | M/G2       | G2     |
|-------------------|------------|------------|------------|------------|--------|
| HeLa cells        |            |            |            |            |        |
|                   |            |            |            |            |        |
| Mps1              | 5.7 ± 1.4 × 10^8 | 6.9 ± 1.5 × 10^8 | 1.1 ± 0.46 × 10^2 | 9.2 ± 5.5 × 10^9 | 7.9 ± 5 × 10^4 |
| Cyclin B          | 9.3 ± 3.8 × 10^7 | 1.4 ± 0.6 × 10^6 | 1.6 ± 0.7 × 10^6 | 0.61 ± 0.02 × 10^1 | N/A    |
| Cdk1              | 2.1 ± 0.5 × 10^6 | 2.4 ± 0.6 × 10^6 | 2.7 ± 0.6 × 10^4 | 2.6 ± 0.6 × 10^6 | 2.1 ± 1.2 × 10^5 |

Concentration (nM)

|          |            |            |            |            |        |
|----------|------------|------------|------------|------------|--------|
| Mps1     | 36 ± 8.7   | 41 ± 6.8   | 61 ± 16    | 46 ± 16    | 34 ± 4.3 |
| Cyclin B | 5.9 ± 0.2 × 10^7 | 8.7 ± 0.4 × 10^2 | 10 ± 0.5 × 10^2 | 3.9 ± 1.3 × 10^2 | N/A    |
| Cdk1     | 13.4 ± 3.3 × 10^2 | 15.2 ± 3.6 × 10^2 | 17.4 ± 3.6 × 10^2 | 1.7 ± 3.8 × 10^2 | N/A    |

Table 1: Quantification of absolute Mps1, cyclin B, and Cdk1 cellular concentrations in HeLa cells

N/A, not applicable.

The carboxyl-terminal tail of Mps1 is critical for substrate phosphorylation. To understand how Mps1 transmits checkpoint signaling, we sought to identify regions of Mps1 that might be involved in substrate recruitment. Crystallographic analysis of Mps1 indicated that whereas the kinase domain in the carboxyl-terminal region of Mps1 adopts a canonical kinase fold, the 65-residue carboxyl-terminal tail of Mps1 is unstructured (23, 29) (Fig. 2A). To decipher a potential function of this region in Mps1, we removed the last 65 residues from Mps1 and expressed the resulting mutant (Mps11–516) in insect cells using the baculovirus expression system (Fig. 3). We expressed and purified wild type and mutant Mps1 that were void of the last 65 amino acids in insect cells using the baculoviral expression system (Fig. 3A). Wild type and mutant Mps1 were expressed as GST fusion proteins with the GST tag at the amino terminus and a His6 tag at the carboxyl terminus to facilitate purification of the full-length enzymes. A PreScission protease cleavage site was also introduced adjacent to the GST tag. Incubation of the fusion proteins with the protease allows removal of GST tag. Unless otherwise specified, all subsequent studies were performed with Mps1 enzymes without a GST tag but containing a His6 tag at the carboxyl terminus.

To find a suitable substrate for our kinetics studies, we tested several known and novel Mps1 substrates including MBP, Smad2, Mps1-NTD, and SUMO-Mps1-NTD (Fig. 3). Although MBP is a good substrate for Mps1, there is certain degree of substrate inhibition that makes MBP unsuitable for steady state kinetics studies (Fig. 3B). In contrast, Mps1-NTD, an amino-terminal fragment of Mps1 (amino acids 1–270) contains several well documented Mps1 autoprophosphorylation sites (25, 31–33) and is an excellent substrate for Mps1 with no apparent substrate inhibition. SUMO-Mps1-NTD behaves identically to Mps1-NTD, suggesting that the presence of SUMO tag does not affect the substrate properties of Mps1-NTD for Mps1 (Fig. 3C). Because the SUMO tag makes it much easier to express and purify recombinant SUMO-Mps1-NTD from E. coli, we used SUMO-Mps1-NTD as the substrate in subsequent studies for Mps1 kinetics analysis.

A detailed reaction time course with wild type Mps1 revealed that product formation was not linear (supplemental Fig. 1). Such a kinetic lag phase is frequently observed with protein kinases and may suggest that autophosphorylation is required to elevate protein kinase activity. To test this hypothesis, we preincubated Mps1 with cold ATP for 40 min before the addition of substrate. As shown in supplemental Fig. 1, the enzyme assay now produced a linear time course.
The observed time course also showed that Mps1 activity was regulated by autophosphorylation as the exhibited activity was 2–3-fold greater after preincubation. This result further confirmed that autophosphorylation of Mps1 increases kinase activity and is consistent with the previous observations that autophosphorylation of the activation and the p + 1 loop elevates kinase activity in vivo and in vitro (23, 31–34).

Additional experiments were performed to investigate changes in the reaction rate as a function of input enzyme concentrations and to determine whether there is product inhibition in Mps1 kinase reaction. The rate of production formation increases linearly with an increase in enzyme concentration within the 15 min of incubation time, suggesting that even at 200, 300, 400, or 500 nm enzyme concentration, the reaction is not saturated with the substrate provided. Sampling at intervals of 5 min within a 30-min period is likely to provide good estimate of initial velocity because the production of product is linear with time during the course of the time interval used (supplemental Fig. 1).

To further validate that the carboxyl-terminal tail of Mps1 is crucial for transphosphorylation of the substrate in a fully reconstituted system, we measured substrate and autophosphorylation activity of purified Mps1 and Mps1ΔD using Mps1-NTD and GST-borealin as the substrates. Recent studies demonstrated that borealin is a substrate of Mps1 and that its phosphorylation is critical for regulating the activity of the Aurora B kinase (16, 35). Borealin and Aurora B are part of the chromosome passenger complex that regulates chromosome alignments and congression. As shown in Fig. 3, D and E, the wild type Mps1 displays robust phosphorylation of both substrates, whereas Mps1ΔD is less efficient in phosphorylating Mps1-NTD and GST-borealin despite an identical input amount of Mps1 and Mps1ΔD, as verified by immunoblotting with an Mps1 antibody that recognizes the amino terminus of Mps1 (Fig. 3E). Compared with transphosphorylation, autophosphorylation activity of Mps1 and Mps1ΔD is very similar in this time course experiment (top bands in Fig. 3E). These results indicate that the carboxyl-terminal tail of Mps1 is critical for transphosphorylation of the substrate and largely dispensable for autophosphorylation of Mps1, suggesting that transphosphorylation and autophosphorylation have different requirements for engaging substrates. Thus, these two phosphorylation processes are at least partially separable in Mps1.

FIGURE 2. The carboxyl-terminal tail of Mps1 is required for substrate phosphorylation. A, structure representation of Mps1 kinase domain (PDB code 3DBQ). The dashed line illustrates the unstructured carboxyl-terminal tail of Mps1 (792–857). B, removal of the carboxyl-terminal tail of Mps1 impairs Mps1 phosphorylation of Smad2 in 293T cells. Wild type, kinase dead, and deletion mutants of Mps1 were cotransfected with FLAG-Smad2 in 293T cells. Forty-eight hours after transfection, cell lysates were prepared and blotted for Mps1 (Mps1NT), Smad2 (FLAG), phospho-Smad2 (pSmad2), and phospho-Mps1 (phospho-Thr-686). C, shown is an immunoprecipitation kinase assay of Mps1 and Mps1 mutants. Myc-tagged wild type and mutant Mps1 were expressed in 293T by transient transfection. Forty-eight hours after transfection, the wild type and Mps1 mutant proteins were immunoprecipitated using a Myc antibody and incubated with 1 μg of MBP in the presence of [γ-32P] ATP under the in vitro kinase reaction conditions described under “Experimental Procedures.” GST-Mps1 purified from insect cells is used as a control. The kinase reactions were terminated by adding 2× SDS sample buffer, and proteins were resolved by 10% SDS-PAGE before phosphorimaging analysis. The amount of Mps1 and Mps1 mutants was determined by immunoblotting (WB) using an anti-Myc antibody. The input substrate MBP was visualized by Coomassie Blue staining. D, shown is a schematic representation of the Mps1 domain organization and expression vectors used in C.
Steady State Kinetics of Mps1 and Mps1ΔD—Having established a suitable substrate for Mps1 kinetics study, we determined the kinetic mechanism of the phosphorylation reaction and the effect of deleting the carboxyl-terminal tail (Mps1ΔD). The entire dataset for each enzyme was fit to the equations for both a random sequential and ping-pong mechanism (see Equations 2 and 3 under “Experimental Procedures”) to determine the best fit. In both cases, the sequential mechanism could accurately fit the data, but the ping-pong mechanism did not. Double-reciprocal plots gave a series of intersecting lines (Fig. 4, A and B), consistent with formation of a Mps1-ATP-Mps1-NTD ternary complex. If the reactions followed the ping-pong mechanism, the plots should have been parallel lines. The lines did not converge exactly on the x axis, suggesting that binding of one substrate affects the binding of the other substrate, i.e. the two substrate binding sites interact. In addition, the two-site interaction factor of 2 suggests that the binding of one substrate decreases the affinity of the enzyme for the other substrate.

Table 2 summarizes the kinetic constants for Mps1 and Mps1ΔD. Deleting the carboxyl terminus of Mps1 increases the $K_m$ of the protein substrate by around 2.5-fold, whereas this deletion has little effect on the $K_m$ of ATP (74 and 80 μM). Loss of the carboxyl terminus from Mps1 also decreases $k_{cat}$ by a factor of 2.3, indicating that the carboxyl terminus is required for optimal catalysis. The combined effects on $K_m$ and $k_{cat}$ result in Mps1ΔD having a 6-fold lower catalytic efficiency than Mps1 ($k_{cat}/K_m$). Furthermore, the minimal effects on the $K_m$ for ATP but much larger effects on $k_{cat}$ and the $K_m$ for protein substrate suggest that the carboxyl terminus of Mps1 interacts with the protein substrate to enhance catalysis.

Intermolecular Versus Intramolecular Autophosphorylation of Mps1—Previous investigations suggest that autophosphorylation of Mps1 can proceed through an intermolecular mechanism as observed in wild type Mps1 phosphorylating a kinase dead Mps1 (23, 32, 33). To directly determine whether one molecule of catalytically active Mps1 can phosphorylate another catalytically active Mps1 and that this pathway of phosphorylation is more rapid than a single molecule of Mps1 phosphorylating itself, we measured autophosphorylation at different concentrations of wild type Mps1. If autophosphorylation were largely an intramolecular reaction, we would expect the rate of autophosphorylation to be independent of concentration. On the other hand, if autophosphorylation were intermolecular, the rate of phosphorylation will vary with the input concentration. Fig. 5, A and B, shows that the rate of autophosphorylation increases with increasing concentrations of Mps1, consistent with the hypothesis that autophosphorylation of Mps1 is largely an intermolecular reaction.

The Carboxyl Terminal Tail of Mps1 Is Critical for Spindle Assembly Checkpoint Activation in Response to Spindle Poisoning—Mps1 is required for activation of spindle assembly checkpoint in response to treatment with spindle disrupting chemicals such as nocodazole. If the carboxyl-terminal tail of Mps1 is engaged in substrate recruitment in cells, we would expect that the removal of this region should affect the checkpoint function of Mps1. To test this hypothesis, we stably expressed siRNA insensitive Mps1 and Mps1ΔD in SW480 cells at close to the endogenous levels. The siRNA-insensitive scramble mutations were described previously (25). Control, wild type (FTH-Mps1R) and mutant Mps1 (FTH-Mps1R ΔΔ) cells were treated with control (luciferase) and Mps1 siRNA.
Although the FLAG-His\(^{6}\)-tagged Mps1 is slightly larger than the endogenous Mps1, the tagged deletion mutant co-migrates with the endogenous Mps1 (Fig. 6A). Transfection with Mps1 siRNA yielded a reduction of the endogenous Mps1 that was not observed in control siRNA. Ectopically expressed Mps1 and Mps1 mutant are unaffected by either siRNA, which is further confirmed by FLAG immunoblotting (Fig. 6A, middle panel). These three cell lines were first synchronized by double thymidine treatment followed by releasing into nocodazole-containing media for 12 h. As shown in Fig. 6B, depletion of Mps1 in control cells abrogates prometaphase arrest. Cells expressing siRNA-insensitive epitope-tagged wild type Mps1 undergo prometaphase arrest as expected with control or Mps1 siRNA treatment. However, cells expressing mutant Mps1 failed to sustain prometaphase arrest in the presence of nocodazole when treated with Mps1 siRNA. This result suggests that the carboxyl-terminal tail of Mps1 is required for spindle checkpoint activation.

### DISCUSSION

Mps1 is primarily a mitotic kinase whose abundance and activity are elevated at the G\(_2\)/M boundary. Using quantitative immunoblotting, we estimated the absolute abundance of Mps1, Cdk1, and cyclin B1 during cell cycle progression in HeLa cells. The levels of all three proteins peak in mitosis at concentrations of 0.06, 1.75, and 1 \(\mu\)M, respectively. We have also investigated the biochemical and kinetic mechanisms of Mps1 kinase. The structurally disordered carboxyl-terminal tail (~65 amino acids) of Mps1 is critical for substrate transphosphorylation both in vitro and in cultured cells. Mps1

**TABLE 2**

Enzyme kinetic constants from the two substrate kinetic analysis

Uncertainties represent 95% confidence intervals from the fitting procedure. Parameters are derived from the global, nonlinear best fit of the velocity data to the ternary complex model [Equation 2].

|           | \(K_{\text{m, Mps1-NTD}}\) | \(K_{\text{m, ATP}}\) | \(V_{\text{max}}\) | \(k_{\text{cat}}\) | \(k_{\text{cat}}/K_{\text{m, ATP}}\) | \(K_{\alpha}\) |
|-----------|---------------------------|---------------------|------------------|----------------|---------------------------------|---------------|
| Mps1 WT   | 54 ± 9 \(\mu\)M           | 74 ± 11              | 9.5 ± 0.8        | 95             | 1.8                             | 37 ± 10       |
| Mps1 ΔD   | 130 ± 18 \(\mu\)M         | 80 ± 14              | 4.2 ± 0.6        | 41             | 0.3                             | 34 ± 14       | 2.3 ± 0.9
autophosphorylation, a priming event for its activation, mostly occurs through an intermolecular mechanism. Removal of the unstructured tail of Mps1 inactivates its spindle checkpoint function.

Although the abundance of Mps1 in mammalian cells has not been previously investigated, other studies have measured cyclin B1 or Cdk1 in HeLa cells (36–38), mouse (39), Xenopus (40), and yeast (41). There were some disagreements in the literature regarding the concentrations of cyclin B in HeLa cells. Arooz et al. (37) reported the cyclin B1 concentration as low as 0.004 μM in mitotic HeLa cells, whereas others found that cyclin B1 levels between 1.8 μM (38) to 2.9 μM (36) in the same cell line. The cyclin B1 levels in mouse oocyte cells were found to fall between 0.2 and ~0.6 μM (39). Our results found cyclin B1 concentrations of ~1 μM, which is in agreement with these latter studies. The Cdk1 abundance measured in our study (1.75 μM) is close to what Arooz et al. (37) reported (~0.63 μM) but differs somewhat from the levels of Cdk1 reported for mouse oocyte (0.3–0.1 μM) (39). Xenopus egg extracts contain ~50 nM Mps1 (10), a concentration in excellent agreement with our measurements in mitotic HeLa cell extracts. Our results suggest that at the peak of mitosis Cdk1 is only in moderate excess of cyclin B1 in HeLa cells. This finding is in stark contrast to studies in Xenopus extracts, where a vast excess of Cdk1 is observed over cyclin B1 (10–100-fold). Both Cdk1 and cyclin B1 are 10–20-fold more abundant than Mps1 in HeLa cells, whereas the concentrations of Mps1 and Cdk1 are very similar in the Xenopus egg extracts. Differences in relative abundance of these key cell cycle regulators may contribute to some of the major differences in cell cycle control between somatic cells and embryo cells (42).

Accumulating evidence suggests that Mps1 is a key component of the spindle checkpoint signaling cascade and functions upstream of all known Mad and Bub gene products (1). A thorough understanding of the properties of this complex signaling pathway and its ability to mediate robust mitotic arrest depends on our ability to quantify the absolute number of each component in the pathway as well as an understanding of how they are integrated. The abundance of checkpoint components is of particular importance, as both over- or under-expression of these components can lead to aneuploidy and subsequent carcinogenesis (1).

The concentrations of Mps1 are tightly controlled by cell cycle progression, with rising levels at the transition from G2 to M and declining as the cell progresses into anaphase. In yeast, Mps1 is degraded via APCcdh1-mediated proteolysis, which is critical for mitotic exit (43). It will be interesting to determine whether this mechanism is also involved in regulating the abundance of mammalian Mps1. Previous studies have estimated the abundance of a few checkpoint proteins including Mad2, BubR1, and Cdc20 in HeLa cells at 120, 90,
and 100 nM, respectively (44). Future studies are needed to quantify the abundance and dynamic range of all checkpoint proteins during the cell cycle. Although the entire dataset is still incomplete, a theme does begin to emerge from our results and those in the literature. It appears that at least in mammalian systems the absolute abundance of the spindle checkpoint components falls in a similar range (~100 nM) and is 10–20-fold less abundant than Cdk1/cyclin B, which is the ultimate target of the spindle checkpoint.

The carboxyl terminus of Mps1 is disordered in both of the reported Mps1 crystal structures (23, 29). The carboxyl terminus of Mps1 does not appear to be required for its autophosphorylation but is critical for transphosphorylation of the substrates (Fig. 3, D and E). Because both reactions are intermolecular, this implies that different binding interfaces are utilized in autophosphorylation versus substrate phosphorylation. The effect of deletion of the carboxyl terminus of Mps1 on substrate phosphorylation is more pronounced in cells than in vitro. Potentially, this may have resulted from the different in relative ratios of Mps1 to its substrate in cells versus in vitro. In cells the amount of Mps1 expressed is about the same as its substrate Smad2. Moreover, in a cellular environment there are many other cellular Mps1 substrates and allosteric regulators present. Finally, phosphatases that could counter the activity of Mps1 may also have an effect. All of these factors could contribute to differences observed between in vitro and in vivo Mps1 activity.

The most straightforward interpretation of the impact of the carboxyl-terminal tail of Mps1 on substrate phosphorylation is that this domain is directly involved in contacting the substrate. However, we cannot rule out an allosteric role of this region during kinase activation and substrate recruitment. In this vein, the tail could stabilize the active conformation of Mps1 and expose the substrate binding site. Determining the crystal structure of Mps1 with its cognate substrate will help to resolve the exact mechanism by which the carboxyl-terminal tail regulates Mps1 activity.

Mps1 is a critical kinase, and its activity is required for both normal chromosome alignment and for regulating the spindle checkpoint. Consistent with restricted activity in mitosis, expression of Mps1 is strongly associated with cell proliferation such that Mps1 levels are markedly reduced or absent in resting cells and in tissues with a low proliferative index (45). Elevated levels of Mps1 are found in a variety of human cancers including thyroid papillary carcinoma, gastric cancer, and bronchogenic carcinoma (5, 46, 47). Complete inactivation of the spindle checkpoint results in cell death due to massive chromosome segregation errors and mitotic catastrophe (48, 49). Accordingly, ablation of Mps1 activity could have therapeutic potential in cancer treatment (50). A recent study suggests that partial inhibition of Mps1 makes tumor cells more sensitive to clinical doses of taxol (22). Although small molecule inhibitors of Mps1 have been reported (21, 51–53), the in vivo potency and specificity of these inhibitors need to be improved for them to be useful as potential clinical drugs. Given the unique and important function of the carboxyl-terminal tail in Mps1 transphosphorylation, small molecule inhibitors that target the carboxyl terminus of Mps1 could specifically interfere with its function. Such inhibitors might be used alone or in conjunction with other therapeutics (e.g. taxol) to selectively kill tumor cells.

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