The dual-specificity protein kinase Mps1 (monopolar spindle 1) is a phosphoprotein required for error-free mitotic progression in eukaryotes. In the present study, we have investigated human Mps1 phosphorylation using combined mass spectrometric, mutational and phosphospecific antibody approaches. We have identified 16 sites of Mps1 autophosphorylation in vitro, several of which are required for catalytic activity after expression in bacteria or in cultured human cells. Using novel phosphospecific antibodies, we show that endogenous Mps1 is phosphorylated on Thr686 and Ser821 during mitosis, and demonstrate that phosphorylated Mps1 localizes to the centrosomes of metaphase cells. Taken together, these results reveal the complexity of Mps1 regulation by multi-site phosphorylation, and demonstrate conclusively that phosphorylated Mps1 associates with centrosomes in mitotic human cells.

Key words: antibody, centrosome, kinase, mitosis, monopolar spindle 1 (Mps1), phosphorylation.

INTRODUCTION

Mps1 (monopolar spindle 1) is a cell-cycle-regulated protein kinase with an important catalytic role in maintaining the SAC (spindle assembly checkpoint), which prevents premature anaphase in eukaryotes [1]. Experimental manipulation of Mps1 function perturbs the SAC [2,3] and induces chromosome segregation defects [4]. Since aneuploidy is a common trait in cancer cells, dissecting the Mps1 regulatory mechanism is important for understanding cancer biology.

As cells enter mitosis Mps1 becomes hyperphosphorylated and activated and is then dephosphorylated at around the time of mitotic exit [3,5]. Several sites of Mps1 autophosphorylation have been reported, including Thr676 in the Mps1 activation loop, which regulates Mps1 catalytic activity and is important for normal mitotic progression in eukaryotic cells [6–8]. Mps1 has also been demonstrated to be a target for the proline-directed kinases Cdk2 (cyclin-dependent kinase 2) and MAPK (mitogen-activated protein kinase) in several model eukaryotes [9,10], where Mps1 phosphorylation might control subcellular distribution during the cell cycle.

Centrosomal Mps1 localization has been demonstrated in human cells, suggesting that a centrosome-dependent function might be conserved between fungi and metazoans [5,9,11]; however, independent studies have failed to find evidence for centrosomal Mps1 [3,12], and this proposed localization is therefore controversial.

In the present study, we have employed a powerful combination of CID (collision-induced dissociation) and ETD (electron-transfer dissociation)-based MS/MS (tandem MS) to show that the Mps1 catalytic domain autophosphorylates on at least 16 residues in vitro, many of which represent novel phosphoacceptor sites. Using a mutagenic approach, we have analysed the importance of these residues for Mps1 activation by autophosphorylation after expression in both bacteria and mitotic human cells. Additionally, we have evaluated several novel Mps1 phosphospecific antibodies, two of which we exploit to demonstrate for the first time that endogenous phosphorylated Mps1 localizes to the centrosomes during mitosis.

EXPERIMENTAL

Molecular biology and protein expression

The human Mps1 catalytic domain (encoding residues 510–857), was amplified by PCR and inserted into the plasmid pET-30 Ek/LIC (Novagen) for bacterial expression. Full-length Mps1 (encoding residues 1–857) was cloned into the vector pBAC-2cp (Novagen) for production in Sf9 cells or into pcDNA5/FRT/TO Invitrogen that had been engineered to include GFP (green fluorescent protein). Mps1 was mutated by PCR to generate kinase-dead (D664A) or phosphorylation-site mutations. All constructs were sequenced over the entire coding region.

Phosphorylation-site mapping by MS

His-tagged Mps1 (residues 510–857) was purified by affinity chromatography, dialysed into 50 mM ammonium bicarbonate (pH 8.0), and then reduced, alkylated and trypsinized prior to LC-MS/MS (liquid chromatography-MS/MS) analysis on a Q-Tof I instrument (for CID) or an HCTultra instrument (for ETD). The centroided peak lists of the CID and ETD spectra were searched against the Mps1 protein sequence using MASCOT, with carbamidomethyl cysteine set as a fixed modification, and methionine oxidation and phosphorylation on serine, threonine and tyrosine as variable modifications. The CID and ETD spectra were subjected to manual inspection to confirm phosphorylation sites.

Abbreviations used: CID, collision-induced dissociation; ETD, electron-transfer dissociation; GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; Mps1, monopolar spindle 1; MS/MS, tandem MS; SAC, spindle assembly checkpoint; siRNA, small interfering RNA; WT, wild-type.

† To whom correspondence should be addressed at the present address: Yorkshire Cancer Research (YCR) Institute for Cancer Studies, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield S10 2RY, U.K. (email p.eyers@sheffield.ac.uk).

‡ Michael Barber Centre for Mass Spectrometry, Manchester Interdisciplinary Biocentre, School of Chemistry, University of Manchester, Manchester M1 7DN, U.K.
Supernatants were prepared prior to Western blotting as previously
or incubated with nocodazole for 12 h prior to lysis. Cleared

Table 1 Summary of Mps1 autophosphorylation sites identified by CID and ETD MS/MS

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<th>Phosphopeptide sequence</th>
<th>Phosphorylation site(s)</th>
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<th>Observed mass (Da)</th>
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Protein kinase and phosphatase assays
For protein kinase and phosphatase assays, 500 ng of purified bacterially expressed Mps1 catalytic domain proteins or GFP–Mps1 protein immunoprecipitated from 200 μg of HeLa cell lysate were assayed by autophosphorylation (GFP–Mps1), together with the exogenous substrate MBP (20 μg; myelin basic protein) or using MBP alone for 30 min at 30°C, essentially as described previously [13]. Phosphatase experiments were performed as previously reported [13].

Antibodies
Sheep anti-(Aurora A) and anti-Bub1 antibodies were provided by Stephen Taylor (Faculty of Life Sciences, University of Manchester, Manchester, U.K.). A monoclonal anti-GFP antibody was from Cancer Research UK. Anti-γ-tubulin (clone GTU-88) was from Sigma. A monoclonal C-terminal pan-Mps1 antibody that recognizes phosphorylated and dephosphorylated Mps1 equally well (Figure 3A) was purchased from Millipore. Fluorescent secondary antibodies were from Jackson Immunoreagents. Phosphospecific Mps1 antibodies were generated in rabbits (Eurogentec) and purified and neutralized with immunizing peptides using standard procedures.

Cell culture, synchronization and transfection
GFP–Mps1 plasmids or Mps1 siGENOME ON-TARGET plus, or Lamin A/C siRNA duplexes (Dharmacon) were transiently transfected into HeLa cells using LipofectamineTM 2000 according to the manufacturer’s instructions (Invitrogen), prior to fixation, or incubated with nocodazole for 12 h prior to lysis. Cleared supernatants were prepared prior to Western blotting as previously described [14]. HeLa cells were fixed in methanol at −20°C, and stained using standard immunofluorescence protocols. For synchronization, HeLa cells were cultured and arrested using a standard double-thymidine block-and-release protocol [14].

RESULTS AND DISCUSSION
Phosphorylation site analysis by MS
Human Mps1 was initially characterized as a phosphoprotein after expression and isolation from bacteria [15,16]; however, the extent of Mps1 catalytic domain phosphorylation in vitro has remained unclear. We focused our initial studies on bacterially expressed human Mps1 containing the kinase domain and its C-terminal extension, which is soluble and catalytically active after affinity purification [13]. Bacteria represent an attractive model organism for investigating Mps1 autophosphorylation, since the active enzyme is generated in the absence of human protein phosphatases, permitting the isolation of large amounts of kinase phosphorylated to high stoichiometry. Consequently, we exploited a combination of CID- and ETD-based MS/MS to identify the sites of Mps1 phosphorylation after expression in bacteria (Table 1). In contrast with CID, ETD fragmentation preserves the phosphate group on the modified amino acid, aiding identification of the site(s) of phosphorylation. We obtained overall Mps1 sequence coverage of 87%, and definitive evidence for 16 phosphorylation sites, the majority of which were validated internally using the two complementary methods of peptide fragmentation (Table 1). The fully annotated MS/MS spectra of the Mps1 phosphopeptides obtained after fragmentation are presented in Supplementary Figures S1–S3 (at http://www.BiochemJ.org/bj/417/bj4170173add.htm). The documented Mps1 phosphorylation sites exhibit a high degree of conservation between model vertebrate species (Figure 1A), although they map to different regions of the human kinase domain, including several residues in the activation segment located between Asp664 and Glu693 (Figures 1B and 1C). We also identified a cluster of Mps1 autophosphorylation sites located outside the catalytic domain, including Ser521, the human equivalent of the previously reported Xenopus MAPK site Ser464 [10]. A D664A kinase-dead Mps1 catalytic domain mutant was analysed in an identical fashion, but no phosphopeptides were detected, demonstrating that all of the identified sites were due to Mps1 autophosphorylation (results not shown).

Biochemical analysis of Mps1 kinase activity in vitro
We mutated 11 of the most conserved Mps1 phosphorylation sites to alanine, to prevent phosphorylation, or aspartate, to attempt to mimic phosphorylation, and assayed their activity after affinity purification from bacteria. The WT (wild-type) Mps1 catalytic domain was active towards MBP, and this served as a standard for comparative analysis of each Mps1 mutant. The bacterially expressed WT catalytic domain did not autophosphorylate to any great extent in the presence of [γ-32P]Mg-ATP,
Mps1 phosphorylation site analysis

Figure 1 Mps1 structure comparison

(A) Conservation of Mps1 autophosphorylation sites determined in the present study. Amino acid usage at the equivalent site is detailed for three model eukaryotic species. (B) Mps1 activation segment sequences (human numbering) from selected species are aligned between kinase subdomains VII and VIII. Also detailed is part of the Mps1 C-terminal extension, which only appears to be conserved in vertebrate genomes. This segment contains several in vitro Mps1 autophosphorylation sites, including Ser821. Hs, Homo sapiens; Mm, Mus musculus; Xl, Xenopus laevis; Dr, Danio rerio; Dm, Drosophila melanogaster; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe. (C) Location of autophosphorylation sites mapped on a ribbon diagram determined from the crystal structure of the Mps1 catalytic domain. Selected autophosphorylation sites are shown in ball and stick representation. Thr564, Ser582 and Ser742 are located outside the activation segment.

presumably because it had already become highly autophosphorylated in bacteria (Figure 1); we were therefore unable to use this parameter to assess the activity of Mps1 in a kinase assay. Kinase-dead D664A Mps1 was catalytically inactive and exhibited an enhanced electrophoretic mobility by SDS/PAGE compared with WT, indicating that autophosphorylation of the kinase in bacteria is responsible for the reduced mobility of the WT enzyme. As shown in Figure 2(A), mutation of Thr675 to alanine increased Mps1 catalytic domain activity, and this was reduced to WT levels by mutation to aspartate, suggesting that a negative charge at this position might be important to maintain WT Mps1 levels of activity. The mobility of T675A was reduced when compared with the WT enzyme, and this shift was reversed in the T675D mutant, verifying that activity correlates positively with decreased gel mobility. The mutation of Thr676 to alanine reduced catalytic activity markedly, and this mutant exhibited high gel-mobility. Interestingly, neither Mps1 activity, nor the associated increased gel mobility, could be rescued by mutation to aspartate, suggesting either that autophosphorylation on this T-loop site is not mimicked by aspartate, or that Thr676 might have a structural role in catalysis. Given the importance of phosphorylation at the equivalent T-loop site for full kinase activity in the protein kinase superfamily [17], we favour the former model, although a high-resolution crystal structure of Mps1 phosphorylated on Thr676 will be needed to prove that the phosphorylation of this amino acid does indeed regulate activity. Interestingly, even among protein kinases that have been verified by structural means to be regulated by phosphorylation at the T-loop residue, such as Aurora A and B, introduction of negatively charged amino acids at this position does not always rescue catalytic activity in vitro [18,19]. As shown in Figure 2(A), Thr686 was indispensable for kinase activity, because the fast migrating, completely inactive alanine mutant could not be rescued by mutation to either aspartate or glutamate (results not shown). This suggests that this residue is either critically required for the structural integrity of the kinase, or is an important phosphoacceptor site that cannot be mimicked by negatively charged amino acids. Analysis of the dephosphorylated Mps1 crystal structure reveals that this amino acid lies in the P+1 loop of the kinase (Figure 1C), pointing towards a dominant structural role for this residue in either substrate binding or ATP positioning during catalysis [13]. This does not, however, rule out an additional regulatory role for the phosphorylation of this residue.

Interestingly, autophosphorylation outside the activation segment was also important for activity in vitro, since S582A/S582D and Y811F mutants exhibited decreased activity, and an S742A mutation led to Mps1 inactivation, which was completely restored by mutation to aspartate (Figure 2A). T806D Mps1 was significantly less active than T806A, demonstrating a potential negative correlation between phosphorylation and activity at this
After immunoprecipitation from nocodazole-treated HeLa cells. A GFP immunoblot demonstrates similar recovery of GFP–Mps1 in the immunoprecipitates (top panel). Immunoprecipitates were assayed with MBP and experiments. The molecular mass (in kDa) is indicated on the left-hand side.

SDS/PAGE. Incorporation of ATP into MBP was linear over the course of the assay. (A) N-terminally GFP-tagged Mps1, or the indicated Mps1 mutants (all full-length), were transfected and assayed after immunoprecipitation from nocodazole-treated HeLa cells. A GFP immunoblot demonstrates similar recovery of GFP–Mps1 in the immunoprecipitates (top panel). Immunoprecipitates were assayed with MBP and [γ-32P]Mg-ATP and 32P incorporation was visualized by autoradiography after SDS/PAGE. Incorporation of ATP into MBP was linear over the course of the assay. (B) N-terminally GFP-tagged Mps1, or the indicated Mps1 mutants (all full-length), were transfected and assayed after immunoprecipitation from nocodazole-treated HeLa cells. A GFP immunoblot demonstrates similar recovery of GFP–Mps1 in the immunoprecipitates (top panel). Immunoprecipitates were assayed with MBP and [γ-32P]Mg-ATP and 32P incorporation into Mps1 (middle panel) or MBP (bottom panel) was visualized after SDS/PAGE and autoradiography. Mock-transfected lysates (−) were used as assay controls to account for background. AAA refers to an Mps1 mutant in which Thr675, Thr682, Ser582 and Ser821 were all mutated to alanine. Similar results were seen in three independent experiments. The molecular mass (in kDa) is indicated on the left-hand side.

Figure 2 Analysis of Mps1 phosphorylation site mutants

(A) The indicated bacterially expressed Mps1 catalytic domains were purified, dialysed, and 500 ng of each protein was separated by SDS/PAGE and immunoblotted with a pan-Mps1 antibody. The kinase activity of 500 ng of the indicated protein was assessed using 20 μg of MBP in the presence of [γ-32P]Mg-ATP, and phosphate incorporation was visualized by autoradiography after SDS/PAGE. Incorporation of ATP into MBP was linear over the course of the assay. (B) N-terminally GFP-tagged Mps1, or the indicated Mps1 mutants (all full-length), were transfected and assayed after immunoprecipitation from nocodazole-treated HeLa cells. A GFP immunoblot demonstrates similar recovery of GFP–Mps1 in the immunoprecipitates (top panel). Immunoprecipitates were assayed with MBP and [γ-32P]Mg-ATP and 32P incorporation into Mps1 (middle panel) or MBP (bottom panel) was visualized after SDS/PAGE and autoradiography. Mock-transfected lysates (−) were used as assay controls to account for background. AAA refers to an Mps1 mutant in which Thr675, Thr682, Ser582 and Ser821 were all mutated to alanine. Similar results were seen in three independent experiments. The molecular mass (in kDa) is indicated on the left-hand side.

Biochemical analysis of Mps1 kinase activity in HeLa cells

To investigate the activity requirements for full-length Mps1 phosphorylation in cells, we transfected GFP-tagged Mps1 constructs containing selected alanine or aspartate mutations, or WT or D664A plasmids as controls. As shown in Figure 2(B), WT GFP–Mps1 was active after immunoprecipitation from HeLa cell extracts, because it readily phosphorylated both itself and MBP. In contrast, D664A GFP–Mps1 was inactive, and served as a negative control to account for assay background. WT GFP–Mps1, but not D664A GFP–Mps1, was detected as a doublet after immunoblotting of the kinase reaction, suggesting that the more slowly migrating band might be due to Mps1-dependent autophosphorylation, representing an independent marker for kinase activity. Thr675 and Thr686 were important for Mps1 activity in cells, because mutation of Thr675 to alanine partially blocked activity, and mutation of Thr686 to alanine completely inactivated the kinase. A triple T-loop alanine mutant was largely inactive, demonstrating conclusively that phosphorylation of one or more of these amino acids is required for full enzymatic function in cells. In addition, the Thr675, Thr686 and triple T-loop alanine mutants all exhibited increased electrophoretic mobility compared with WT, indicating low levels of autophosphorylation and activity. Although mutation of Thr675 to aspartate partially rescued both Mps1 activity and the associated electrophoretic shift, Thr686 aspartate or glutamate (results not shown) mutants were completely inactive in human cells, mirroring our finding that mutation of this residue abolishes Mps1 activity in vitro. Interestingly, both S582A and S582D mutants appeared to be less active than the WT enzyme, whereas S742D and S742E (results not shown) mutants were hyperactive and electrophoretically retarded, suggesting that these two sites might be involved in modulating enzyme activity in cells. None of the other identified phosphoacceptor sites tested, including Thr675, appeared to be critical for Mps1 activity under these conditions, since their gel mobility and activity were similar to that of WT Mps1. As previously reported for Xenopus Mps1 [10], Ser821 phosphorylation was not essential for the activity of human Mps1 in cells, since S821A and S821D mutants both retained WT activity and reduced gel mobility.

We consistently noted a difference in the activity of some Mps1 mutants, depending upon whether they were expressed in bacteria, or immunoprecipitated from human cells, prior to assay under identical reaction conditions with the non-specific substrate MBP. For example, mutation of Thr676 to aspartate markedly decreased Mps1 catalytic domain activity in vitro, suggesting that phosphorylation at this site might decrease activity; however, the same mutation did not negatively influence Mps1 activity after isolation from mitotic cells. In addition, a T675A mutant was hyperactive in vitro, but exhibited similar activity to WT Mps1 after isolation from cells. In contrast, an S742A mutant was inactive when isolated from bacteria, but exhibited WT activity after expression in cells, which was enhanced several-fold by mutation to aspartate or glutamate. We also demonstrated that a Y811F mutant isolated from HeLa cells exhibited kinase activity and a decreased electrophoretic mobility, despite being expressed at much lower levels than other Mps1 proteins (Figure 2B). This suggests, but does not prove, that Tyr811 phosphorylation might be associated with Mps1 stability and/or decreased activity in cells. This is in contrast with the inhibitory effect of abolishing phosphorylation at this site in a Y811F mutation in bacteria (Figure 1A). To explain these differences, we speculate that the folding of the Mps1 catalytic domain mutants might be particularly susceptible to certain amino acid mutations in bacteria, but that these may be counterbalanced or masked by other factors.
Mps1 phosphorylation site analysis

Figure 3 Characterization of phosphospecific antibodies

(A) 500 ng of bacterially expressed WT or D664A Mps1 catalytic domain proteins (amino acids 510–857) were either mock-treated or incubated with λ-phosphatase (λ-PPase). The phosphatase was inactivated with orthovanadate prior to assay in the presence or absence of [γ-32P]Mg-ATP or the substrate MBP. After SDS/PAGE, the proteins were immunoblotted with the indicated Mps1 antibodies and radiolabelled MBP was identified by autoradiography. (B) WT or D664A Mps1 catalytic domain proteins (500 ng) were detected with the indicated phosphospecific Mps1 antibodies that had either been untreated (No PEPTIDE), or pre-incubated with either the immunizing phosphopeptide (+phosPEPTIDE) or the immunizing dephosphopeptide (+PEPTIDE) prior to immunoblotting. (C) The indicated purified bacterially expressed Mps1 catalytic domain mutants were affinity purified, separated by SDS/PAGE and then immunoblotted with the indicated Mps1 antibodies. (D) His-tagged Mps1 or D664A Mps1 (both full-length) were expressed in Sf9 cells and purified by affinity chromatography. Purified protein (500 ng) was separated by SDS/PAGE and blotted with the indicated Mps1 antibody. The molecular mass (in kDa) is indicated on the left-hand side. (E) GFP–Mps1, or GFP–D664A Mps1 (both full-length), were transfected into HeLa cells and then incubated with nocodazole for 12 h prior to lysis. Soluble extracts (20 μg) were separated by SDS/PAGE and immunoblotted with the indicated Mps1 antibodies. Untransfected cells are indicated by (−) and equal Mps1 expression in the lysates was demonstrated by blotting with an anti-GFP antibody. The molecular mass (in kDa) is indicated on the left-hand side.

such as protein phosphatases or molecular chaperones in human cells, leading to different results when full length Mps1 is assayed after immunoprecipitation.

The significance of Thr676 and Thr686 phosphorylation for Mps1 activity in human cells has also recently been recognized by others [6–8], and given the importance of Mps1 kinase activity for the SAC and accurate chromosome segregation in human cells [6–8,20], it will be important to assess the effects of all of our phosphorylation site mutants on physiological Mps1 function in cells. Based on our activity assays, we hypothesize that several of the newly identified phosphorylation sites, including those positioned outside the catalytic domain such as Ser821, might be important for non-enzymatic functions in human cells, such as substrate docking interactions or subcellular targeting.

Analysis of phosphospecific Mps1 antibodies and mechanism of autophosphorylation

To analyse Mps1 autophosphorylation further, we generated four phosphospecific antibodies, designated pThr675, pThr676, pThr686 and pSer821 respectively. The specificity of these antibodies was demonstrated in vitro using WT (active) and D664A (inactive) Mps1 catalytic domain mutants. WT Mps1, but not D664A catalytic domain, was recognized by all four purified phosphospecific antibodies (Figure 3A), whereas the D664A and WT proteins were recognized equally well by a pan-Mps1 antibody. These results validate some of our MS findings and help prove phosphospecificity for each antibody. Phosphospecificity was also demonstrated independently by incubation of the Mps1 catalytic domains with λ-phosphatase (Figure 3A), by specific peptide neutralization experiments (Figure 3B) and by immunoblotting a panel of Mps1 point mutants (Figure 3C).

We also exploited these phosphospecific antibodies to investigate the mechanism of Mps1 autoactivation. Interestingly, the reactivation of Mps1 catalytic activity after phosphatase treatment was accompanied by autophosphorylation of Thr676 and Thr686, but not Thr675 or Ser821 (Figure 3A), reinforcing the strong positive correlation previously identified between phosphate occupancy at Thr676 and Thr686 and Mps1 activity (Figures 2A and 2B). Reactivated Mps1 was only 20% as active towards MBP as the untreated enzyme, and only partially phosphorylated at Thr676 and Thr686 under these conditions (Figure 3A). This suggests that sub-stoichiometric phosphorylation at these sites within the population might limit activity, or that additional phosphorylated amino acids could be required to generate the Mps1 conformation exhibiting full catalytic activity.

The analysis of Mps1 point mutants with phosphospecific antibodies also revealed additional insights into the mechanism
of Mps1 activation in vitro. As shown in Figure 3(C), the pThr<sup>675</sup> antibody failed to recognize phosphorylated Thr<sup>675</sup> in the T686A mutant, but not a T686D Mps1 mutant, suggesting that an aspartate residue at this position might generate sufficient Mps1 activity in bacteria to permit autophosphorylation at Thr<sup>675</sup>. Both T686A and T686D Mps1 mutants contained phosphorylated residues in addition to Thr<sup>675</sup>, including Thr<sup>676</sup> and Ser<sup>821</sup>. This demonstrates that although these mutants exhibit no detectable activity when assayed under rate-limiting assay conditions with MBP in vitro (Figures 2A and 2B), and the same T686A mutant adopts an ‘inactive’ confirmation when crystallized [13], the high enzyme and ATP concentrations in bacteria might promote auto-phosphorylation by this mutant when overexpressed. Importantly, the D664A mutant was not detected by any of the phosphospecific antibodies, either after phosphatase treatment and ATP incubation, or after bacterial synthesis and purification using identical conditions as the WT enzyme.

In addition, these results also provide some evidence for the hierarchical nature of Mps1 autophosphorylation. For example, the finding that Thr<sup>676</sup> and Thr<sup>686</sup>, but not Thr<sup>675</sup> or Ser<sup>821</sup>, became re-phosphorylated in an ATP-dependent manner after phosphatase treatment, suggests that Thr<sup>676</sup> and Thr<sup>686</sup> are preferential sites of autophosphorylation (Figure 3A). Moreover, the finding that Thr<sup>675</sup> and Ser<sup>821</sup> were occupied in the T686A mutant, and that Thr<sup>675</sup> and Ser<sup>821</sup> phosphate occupancy was enhanced many fold in the T686D mutant (Figure 3C), further suggests that the putative phosphomimetic aspartate mutant possesses an increased ability to autophosphorylate in bacteria, explaining why autophosphorylation of Thr<sup>675</sup> and Ser<sup>821</sup> approaches levels found in the WT enzyme. A mixture of MS and high-resolution crystallography of the active, phosphorylated enzyme, will be required to determine how phosphorylation controls Mps1 activity, and to define sites that are necessary and sufficient for Mps1 activity. Such an analysis might also permit some insight into any consensus sequences utilized by Mps1 for directing substrate phosphorylation, which remain unclear despite the emergence of cellular targets in addition to Mps1 [4].

A surprising observation was that the pSer<sup>821</sup> antibody was able to detect the bacterially expressed S821D mutant after electrophoresis. This suggests that the aspartate side chain might be sufficiently phosphomimetic to allow the pSer<sup>821</sup> antibody to recognize the aspartate mutant by Western blotting. Phosphospecific antibodies would not normally be expected to recognize side chains such as aspartate, whose structure would presumably be significantly different to that presented by the phosphorylated amino acid; however, the finding that mutation of Ser<sup>441</sup> of Xenopus Mps1 to aspartate, but not alanine, induces Mps1 localization at kinetochores in egg extracts [10] supports the hypothesis that mimicking the phosphate charge at this site with aspartate might also provide some structural mimicry in this region of the protein.

**Mps1 phosphorylation in eukaryotic cells**

To investigate the phosphorylation status of Mps1 after expression in a eukaryotic cell type, we immunoblotted Mps1 and D664A Mps1 purified from Sf9 insect cells. Although WT and D664A Mps1 were both detected by a pan-Mps1 antibody, the pThr<sup>686</sup> antibody only recognized the WT Mps1 protein. In contrast, the pSer<sup>821</sup> antibody recognized both the WT and D664A mutants (Figure 3D), suggesting that, in contrast with Thr<sup>686</sup>, Ser<sup>821</sup> might not be an autophosphorylation site in cells. To further investigate Mps1 autophosphorylation, we transfected HeLa cells with either GFP–Mps1 or D664A GFP–Mps1, and blotted nocodazole-arrested cells with each phosphospecific antibody. As shown in Figure 3(E), GFP–Mps1 was phosphorylated on Thr<sup>675</sup>, Thr<sup>676</sup>, Thr<sup>686</sup> and Ser<sup>821</sup> in nocodazole-exposed cells. Thr<sup>675</sup>, Thr<sup>676</sup> and Thr<sup>686</sup> phosphorylation was due to Mps1 autophosphorylation, since these sites were absent from the inactive D664A GFP–Mps1 mutant. In contrast, Ser<sup>821</sup> phosphorylation is unlikely to be due to autophosphorylation in human cells, since the pSer<sup>821</sup> antibody also detected phosphorylation on the D664A GFP–Mps1 mutant. These results demonstrate that all four phosphospecific Mps1 antibodies recognize overexpressed Mps1 in human mitotic cell extracts, further validating our in vitro results.

**Mps1 is phosphorylated during mitosis in human cells**

We next examined the phosphorylation of endogenous Mps1 in HeLa cells. We found that Mps1 was phosphorylated at Ser<sup>821</sup> at low levels in asynchronous extracts, but at high levels in nocodazole-treated extracts, suggesting mitosis-specific occupancy of this site. As shown in Figure 4(A), treatment of extracts with γ- phosphatase abolished the pSer<sup>821</sup> signal, demonstrating
Mps1 phosphorylation site analysis

Figure 5  Centrosomal Mps1 is phosphorylated on Ser\textsuperscript{821} and Thr\textsuperscript{686} in HeLa cells

(A) Localization of pSer\textsuperscript{821} Mps1 and Aurora A (top panels) and pSer\textsuperscript{821} Mps1 and Bub1 (bottom panels) in HeLa cells in metaphase of mitosis. Colour images are merged in the right-hand panels.

(B) HeLa cells were transfected with the indicated siRNA duplexes, fixed and co-stained with pSer\textsuperscript{821} and Aurora A antibodies. (C) As in (B), except cells were co-stained with pThr\textsuperscript{686} and Aurora A antibodies. (D and E) Metaphase HeLa cells were stained with pSer\textsuperscript{821} or pThr\textsuperscript{686} Mps1 antibodies that had been pre-incubated with the indicated immunizing peptides prior to analysis. Scale bars = 10 μm.

phosphospecificity against endogenous protein. To prove that this protein was Mps1, and not another protein of a similar electrophoretic mobility, we repressed Mps1 using siRNA. Mps1 levels in asynchronous cells were markedly decreased after Mps1, but not Lamin A/C, siRNA, and this correlated with loss of the pSer\textsuperscript{821} Mps1 signal (Figure 4A).

To assess Ser\textsuperscript{821} phosphorylation throughout the cell cycle, synchronized HeLa cells were released from a double-thymidine block and cell extracts were prepared at different time points. Flow-cytometric analysis demonstrated that the cells synchronously cycled between S-phase, mitosis and G1-phase (results not shown). The mitotic window was between 10 and 13 h after release, as shown by the phosphorylation of Aurora A at Thr\textsuperscript{288} (Figure 4B). As a positive control, we also released cells into nocodazole, which promotes Mps1 activity and arrests cells in mitosis [3]. Total Mps1 protein was detected at all stages of the cell cycle, and it underwent a modest decrease in electrophoretic mobility during mitosis, or after nocodazole exposure, both of which have been shown to correlate with Mps1 activation [3,5].

We next exploited the pSer\textsuperscript{821} antibody to demonstrate that Mps1 was only weakly phosphorylated in S-phase, but became electrophoretically retarded and phosphorylated in mitosis (Figure 4B). To prove that the pSer\textsuperscript{821} antibody was only recognizing Mps1 phosphorylated at Ser\textsuperscript{821}, we performed antibody-blocking experiments with phosphorylated or dephosphorylated immunogenic peptides prior to Western blot analysis (Figure 4B). Taken together, these results show that endogenous Mps1 phosphorylation increases markedly at Ser\textsuperscript{821} during mitosis. Although Ser\textsuperscript{821} phosphorylation does not appear to be required for activity (Figures 1 and 2), this modification coincides precisely with Mps1 activation [3], and it therefore represents a novel marker for mitotic Mps1 in cell extracts.

Phosphorylated Mps1 localizes to centrosomes in mitosis

There is agreement that a fraction of Mps1 is targeted to the kinetochores during mitosis in human cells [3,11]. In contrast, the centrosomal targeting of endogenous Mps1 in human cells is controversial. To investigate this issue further, we exploited two phosphospecific Mps1 antibodies to study Mps1 localization in fixed mitotic HeLa cells, where both endogenous and overexpressed Mps1 has been reported to localize to mitotic centrosomes [5,11]. Consistently, we found that endogenous Mps1 phosphorylated at Ser\textsuperscript{821} co-localized with Aurora A, a known centrosomal protein in mitotic cells (Figure 5A). Co-localization was not detected if pre-immune rabbit serum was
used for analysis, but was evident with the pSer$^{821}$ antibodies if the cells were fixed in methanol or formaldehyde. Furthermore, strong centrosome reactivity was detected in other human cancer cell lines, including U2OS (results not shown) and DLD1 cells (see below). Surprisingly, given the inferred targeting of Mps1 to kinetochores through Ser$^{844}$ phosphorylation in Xenopus [10], we did not find any evidence for Ser$^{621}$ phosphorylation at kinetochores in human cells, although these mitotic structures were readily detected with an anti-Bub1 antibody (Figure 5A).

To prove that the phosphospecific Mps1 antibodies were recognizing Mps1, we employed siRNA to show that the loss of total Mps1, as detected by Western blot analysis of the same transfected cell population (Figure 4A), also led to the loss of centrosome reactivity to the pSer$^{821}$ antibodies by immunofluorescence (Figure 5B), together with impairment of metaphase chromosome alignment, as previously described [8,11]. We next demonstrated that a pThr$^{686}$ Mps1 antibody, which we previously validated using both recombinant and exogenously expressed Mps1 (Figure 3), also specifically stained the centrosome, where it co-localized with the marker Aurora A (Figure 5C). To prove that this antibody was specifically recognizing centrosomal Mps1, we showed that pre-immune rabbit serum did not stain the centrosome (results not shown), and validated that the staining pattern disappeared when Mps1 levels were specifically repressed using siRNA-mediated depletion (Figure 5C). To confirm that both of these antibodies were recognizing Mps1 in a phosphospecific manner, we also demonstrated neutralization of both antibodies by the appropriate phosphorlated immunizing peptides (Figures 5D and 5E).

To investigate intracellular Mps1 localization further, we next demonstrated an interaction between endogenous Mps1 and the well-characterized centrosomal protein γ-tubulin in both fixed cells and cell extracts. Centrosomal γ-tubulin co-localized with pSer$^{821}$ and pThr$^{686}$ Mps1 antibodies in both HeLa and DLD1 cells (Figures 6A and 6B). Furthermore, γ-tubulin antibodies immunoprecipitated Mps1 from nocodazole-treated HeLa cell extracts (Figure 6C). In complementary experiments, the Mps1 antibody also immunoprecipitated γ-tubulin from HeLa cell extracts (results not shown). These results clearly demonstrate that Mps1 localizes to the centrosome in human cells.

Conclusions

The affinity of some polyclonal Mps1 antibodies has been demonstrated to be sensitive to the phosphorylation status of Mps1 [11], providing a potential reason for their reported failure to detect Mps1 at the centrosome [3]. In agreement with this explanation, the pan-Mps1 antibody used in the present study did not detect endogenous Mps1 at kinetochores or centrosomes by immunofluorescence, although we used siRNA to validate that it recognized Mps1 by Western blotting (Figure 3A). In contrast, the pThr$^{686}$ antibody did not detect endogenous Mps1 by Western blotting, but recognized exogenous GFP–Mps1 (Figure 3D) by immunoblotting and endogenous centrosomal Mps1 by immunofluorescence (Figures 6A and 6B). Taken together, these results suggest that antibodies raised against bona fide sites of Mps1 phosphorylation such as Thr$^{686}$ and Ser$^{821}$ might be superior reagents with which to investigate Mps1 function by immunofluorescence. Indeed, the strong correlation between Mps1 activity and the phosphorylation of Thr$^{686}$ (Figure 2A), our discovery that Mps1 is phosphorylated at Thr$^{686}$ on the centrosome indicates that this might represent a catalytically active subcellular pool of the enzyme.

In conclusion, our analysis of the phosphorylation of human Mps1 demonstrates a highly complex pattern of in vitro regulation that is closely mirrored in cells. Phosphate occupancy at Thr$^{676}$, Thr$^{676}$ and Thr$^{686}$ appears to be dependent on Mps1 activity under all conditions studied, and Thr$^{676}$ and Thr$^{686}$ phosphorylation appear to be critically important for Mps1 catalytic activity. In contrast, Ser$^{821}$ phosphorylation is not required for activity, and this modification might be mediated by a distinct proline-directed kinase in eukaryotic systems [10]. We have identified a series of novel Mps1 autophosphorylation sites, several of which map to regions of the catalytic domain outside the activation segment (Figure 1). Although the in vivo function of several of these phosphorylation events is not yet known, we speculate that they may be important for regulating Mps1 autophosphorylation and activity (Figure 2), or subcellular targeting in cells. Our finding that endogenous centrosomal Mps1 is phosphorylated on Thr$^{686}$ and Ser$^{821}$ opens up several avenues for investigating the regulation and function of these specific cellular modifications. These include identification of the human Mps1 Ser$^{821}$ kinase

![Figure 6](image-url)
and dissection of the regulatory requirements for Thr406 and Ser621 phosphorylation for Mps1 activity, targeting and stability at the centrosome. In addition, validated Mps1 phosphospecific antibodies will be invaluable for investigating the intricate regulation of Mps1 by multi-site phosphorylation under a variety of experimental and pathological conditions.

We thank Professor Viki Allan, Professor Philip Woodman, Dr Stephen Taylor and Ms Sonya Hartmuth (University of Manchester) for reagents and advice. We are grateful to Dr Claire Eyers (School of Chemistry, University of Manchester) for expert help with MS analysis and a critical reading of the manuscript before submission. M. L. H. C. acknowledges the University of Manchester School of Medical and Human Sciences for an Overseas Research Studentship, and H. J. acknowledges the EPSRC (Engineering and Physical Sciences Research Council) for a Ph.D. studentship. P. A. E. acknowledges generous funding through an MRC (Medical Research Council) Career Development Fellowship (G120/1030).

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Received 27 June 2008/31 July 2008; accepted 5 August 2008
Published as BJ Immediate Publication 5 August 2008, doi:10.1042/BJ20081310
SUPPLEMENTARY ONLINE DATA
Phosphoregulation of human Mps1 kinase

Rebecca K. TYLER*, Matthew L. H. CHU†, Hannah JOHNSON‡, Edward A. McKENZIE*, Simon J. GASKELL‡ and Patrick A. EYERS*‡

*Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, U.K., †School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester M13 9PT, U.K., and ‡Michael Barber Centre for Mass Spectrometry, Manchester Interdisciplinary Biocentre, School of Chemistry, University of Manchester, Manchester M1 7DN, U.K.

Figure S1 MS/MS spectra of doubly protonated Mps1 tryptic phosphopeptides derived from the Mps1 catalytic domain

The b ions (–) and y ions (–) identified following CID are indicated in both the spectrum and the peptide sequence. The loss of H$_3$PO$_4$ (β-elimination) is indicated by Δ. (A) MS/MS spectrum of LIDFGIANQMQDP(t)PTSVVK. β-elimination at y5 and y6 followed by the double β-elimination from the higher series y ions confirms the presence of phosphorylation at both Thr$^{675}$ and Thr$^{676}$. (B) Spectrum of phosphopeptide LIDFGIANQMQDP(t)PTSVVK. The unmodified y4 and the presence of β-eliminated y5 ion confirms phosphorylation at Thr$^{676}$. (C) Spectrum of singly phosphorylated DSQVG(pT)VNYMPPEAIK. The presence of Δy11 and Δb6 confirms that Thr$^{686}$ is the site of phosphorylation. (D) MS/MS spectrum of TPFQQIINQI(pS)K. The loss of H$_3$PO$_4$ from the ion series starting at y2 confirms the presence of phosphate at Ser$^{742}$. (E) MS/MS spectrum of (pY)VLGQLVGLNSPNSILK. The modified b-series ions and the lack of modification on the y-series ions indicates that Tyr$^{811}$ is the site of phosphorylation. Note that due to their unique chemistry, phosphotyrosine-containing peptides do not lose phosphate by β-elimination. (F) MS/MS spectrum of YVLGQLVGLN(pS)PNSILK. The β-elimination at Ser$^{821}$ (Δy11), indicates that this is the site of modification.

To whom correspondence should be addressed at the present address: Yorkshire Cancer Research (YCR) Institute for Cancer Studies, School of Medicine and Biomedical Sciences, University of Sheffield, Sheffield S10 2RY, U.K. (email p.eyers@sheffield.ac.uk).
Figure S2  CTD MS/MS spectra of Mps1 tryptic phosphopeptides

The b ions (Δ−) and y ions (Δ−) identified following CID are indicated in both the spectrum and the peptide sequence. The loss of H₃PO₄ (β-elimination) is indicated by Δ. (A) Triply protonated YVNLEADNQ(pT)LDSYNEIAYLNK spectrum. The unmodified y₁₃ followed by Δy₁₄ indicates the presence of phosphate at Thr⁵₆₄. (B) Spectrum of singly phosphorylated LQH(pS)DKIIR peptide. The presence of a single serine indicates that Ser⁵₈₂ can be the only phosphorylated residue in this peptide. This is confirmed by β-elimination at y⁷ and y⁸. (C) Spectrum of doubly phosphorylated peptide D(pS)QVG(pT)VNYMPPEAIK. β-elimination throughout the b-series ions from b₂ through to b₅ and double β-elimination at b₆ indicates the presence of phosphate at both Ser⁶₈₂ and Thr⁶₈₆. (D) Spectrum of ISPELLAHPYVQIQ(pT)HPVNOIK. The loss of H₃PO₄ at y₂₁ indicates that the site of phosphorylation is Thr⁷₉₅. (E) G(pT)pT)EEMKYVLGLNSPNIKL spectrum indicating doubly phosphorylated Thr⁸₀₅ and Thr⁸₀₆. (F) Spectrum of YVLGQLVLNSPN(pS)ILK. The modified y₅ and y₆ ions along with the loss of H₃PO₄ at b₁₄ indicates that phosphate is present on residue Ser⁸₂₄.
Figure S3  ETD MS/MS spectra of Mps1 phosphopeptides

The c ions (••), z ions (••) and y ions (••) identified following ETD are annotated in both the spectrum and the peptide sequence. (A) ETD spectrum of LIDFGIANQMQPDT(pT)SVVK. Thr(1776) phosphorylation is confirmed by the presence of z4/z5 and c14/c15 series ions. (B) ETD spectrum of YVLGQLVL(G/N)pS(P)NSILK. Phosphorylation site Ser(821) was confirmed. (C–E) ETD spectra of TLYEHSISGESHNS(pS)SSK, TLYEHSISGESHNS(pS)pTTFEK and TLYEHSISGESHNSpS(pT)TFEK. The absolute site(s) of phosphorylation on these peptides could not be assigned by CID. However, because the phosphate modification remains attached to the peptide backbone during ETD, the site(s) of modification could be accurately determined for these three phosphopeptides.