Phosphorylation regulates nucleophosmin targeting to the centrosome during mitosis as detected by cross-reactive phosphorylation-specific MKK1/MKK2 antibodies

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Phosphorylation-specific antibodies provide a powerful tool for analysing the regulation and activity of proteins in the MAP (mitogen-activated protein) kinase and other signalling pathways. Using synchronized cells, it was observed that phosphorylation-specific antibodies developed against the active form of MKK1/MKK2 (MAP kinase kinase-1 and -2) reacted with a protein that was approx. 35 kDa during G₂/M-phase of the cell cycle. Failure of the 35 kDa protein to react with phosphorylation-independent MKK1/MKK2 antibodies suggested that this protein was not related to MKK1 or MKK2. Thus the 35 kDa protein was isolated by immunoprecipitation with the phospho-MKK1/MKK2 antibody and identified by MS. Peptide sequence analysis revealed matches with NPM (nucleophosmin/B23), a phosphoprotein involved in nucleolar assembly, centrosome duplication and ribosome assembly and transport. Biochemical and immunocytochemistry analyses verified that the phospho-MKK1/MKK2 antibodies cross-reacted with NPM that was phosphorylated at Thr²³⁴ and Thr²³⁷

during G₂/M-phase, which are the same sites that are targeted by Cdc2 (cell division cycle protein-2) during mitosis. Using phosphorylation site mutants, we show that phosphorylation of Thr²³⁴ and Thr²³⁷ is required for NPM immunoreactivity with the phospho-MKK1/MKK2 antibody. Moreover, phosphorylation of Thr²³⁴ and Thr²³⁷ was demonstrated to regulate NPM localization to the centrosome after nuclear envelope breakdown in mitotic cells. These findings reveal a new insight into the role of phosphorylation in regulating NPM targeting during mitosis. However, caution should be used when using commercially available phospho-MKK1/MKK2 antibodies to examine the regulation of MKK1/MKK2 during mitotic transitions, owing to their cross-reactivity with phosphorylated NPM at this time of the cell cycle.

Key words: cell cycle, MAP kinase (mitogen-activated protein kinase kinase), MKK (MAP kinase kinase), mitosis, nucleophosmin, phosphorylation.

INTRODUCTION

Previous studies suggest that the ERK1 and ERK2 (extracellular-signal-regulated kinase-1 and -2) proteins and their upstream activators MKK1 and MKK2 [mitogen-activated protein (MAP) kinase kinase-1 and -2] are involved in regulating G₂/M-phase progression during the cell cycle. For example, inhibition of either MKK1 or MKK2 activity has been attributed to causing alterations in cell-cycle progression through G₂/M-phase [1,2]. For MKK1, stable expression of a dominant-negative form of MKK1 resulted in delayed G₂ progression in 3T3 fibroblasts [2]. MKK2-deficient cells are defective in their ability to undergo cell-cycle arrest in G₂-phase after an ionizing radiation, suggesting a requirement for MKK2 activity in maintaining a G₂ checkpoint [1]. Although specific substrate targets for MKK1/MKK2 and ERK1/ERK2 proteins during G₂/M transitions are not known, ERK1/ERK2 and MKK1/MKK2 may regulate chromosome segregation and organelle disassembly during mitosis. Not only has activated ERK1/ERK2 been shown to associate with kinetochores during early mitosis and to possibly regulate proper metaphase to anaphase transitions [3–5], but MKK1 may also function in regulating disassembly of the Golgi complex during mitotic transitions [6–8].

The development of phosphorylation-site-specific antibodies provides a powerful tool for examining protein regulation by immunoblot and immunohistochemical analyses. Phospho-specific

antibodies raised against ERK1/ERK2 and MKK1/MKK2 have been characterized and used extensively to monitor the activation and localization of these proteins during the cell cycle [3–5,9,10]. In general, these antibodies have been described to be highly specific to the phosphorylation status of the proteins which they target. However, recently, phospho-MKK1/MKK2 antibodies have been reported to recognize a protein of approx. 35 kDa in cells during G₂/M-phase of the cell cycle [4,10]. One study suggested that the 35 kDa protein recognized by the phospho-MKK1/MKK2 antibody was generated by a proteolytic cleavage at the N-terminus of active MKK1 during mitotic transitions [10]. Although the proteolytic activity that was involved in generating the 35 kDa MKK1-related protein was not identified and the identity of this protein was not confirmed by other methods, these findings indicate that partial proteolysis could be a mechanism for inactivating the ERK pathway during G₂/M-phase of the cell cycle [10].

In the present study, we have used MS and biochemical assays to determine whether the 35 kDa protein recognized by the phospho-MKK1/MKK2 antibody during G₂/M-phase is indeed related to MKK1 or MKK2. After immunoprecipitation of the 35 kDa protein from G₂/M-phase-synchronized cell lysates using the phospho-MKK1/MKK2 antibody, MS analysis of associated peptide sequences failed to identify MKK1 or MKK2 in the immunocomplex. Surprisingly, sequence analysis identified NPM (nucleophosmin/B23; also known as NO38), a 33 kDa nucleolar

Abbreviations used: Cdc2, cell division cycle protein-2; Cdk, cyclin-dependent kinase; CIP, calf intestinal phosphatase; DAPI, 4,6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FBS, foetal bovine serum; GST, glutathione S-transferase; HA, haemagglutinin; MAP, mitogen-activated protein; MKK, MAP kinase kinase; NPM, nucleophosmin/B23; TLB, tissue lysis buffer; TMO3, tropomodulin-3; TPM2, tropomyosin-2.

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protein involved in ribosome assembly and chaperone functions [11–13], as a protein that was recognized by the phospho-MKK1/MKK2 antibody during mitotic transitions. We further demonstrate that phospho-MKK1/MKK2 antibodies recognize NPM on the C-terminal region, which is phosphorylated by Cdc2 (cell division control kinase-2) during G₂/M-phase. From these findings, we conclude that commercially available phospho-MKK1/MKK2 antibodies may be useful reagents for monitoring the phosphorylation status of NPM during mitosis. However, these antibodies should be used with caution when interpreting the data associated with MKK1/MKK2 processing and function during mitosis.

MATERIALS AND METHODS

Reagents and cell culture

Cell lines tested in these studies included HeLa, NIH 3T3, IMR90, HEK-293 (human embryonic kidney 293) and Chinese-hamster ovary cells. However, only the data derived from HeLa cells is presented. Cells were grown in DMEM (Dulbecco's modified Eagle's medium) + 10 % (w/v) FBS (foetal bovine serum) as described previously [14]. Phospho-MKK1/MKK2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.; 9121) or Sigma (M7683). The antibodies specific for Cdc2 (#17), Cyclin B1 (GNS1), ERK2 (C-14), the C-terminus of MKK1 (C-18) or MKK2 (C-16) were purchased from Santa Cruz Biotechnology. The N-terminal-specific MKK1 (raised against amino acids 2–124) antibody was purchased from BD Transduction Laboratories (Palo Alto, CA, U.S.A.). Immunoblotting was performed as described in [14]. Staurosporine and roscovitine were purchased from Sigma and Calbiochem (San Diego, CA, U.S.A.) respectively.

HeLa cells were synchronized using a double thymidine block, as described previously [15]. Briefly, cells that were approx. 50 % confluent were treated for 16 h with 2 mM thymidine in DMEM + FBS. Cells were washed with Hanks balanced salt solution and incubated for 8 h in DMEM + FBS in the absence of excess thymidine. Cells were then treated a second time with 2 mM thymidine in DMEM + FBS for 16 h to generate cells arrested at the G₁/S-phase boundary of the cell cycle. Cells synchronized in G₁/S-phase were washed with Hanks balanced salt solution, released back into the cell cycle and harvested at various time points after G₁/S-phase, which corresponded to G₂- and M-phase as determined previously by flow-cytometry analysis [16]. In some cases, cells were arrested in prometaphase of mitosis by treating with nocodazole (100 ng/ml) for 14–16 h. Protein lysates were collected using a rubber scraper in TLB (tissue lysis buffer; 1 % Triton X-100/150 mM NaCl/10 mM Tris, pH 7.4/1 mM EDTA/1 mM EGTA, pH 8.0/0.2 mM sodium orthovanadate/1 mM benzamide/0.2 mM PMSF). After lysis, the lysates were centrifuged at 20 000 g for 10 min to remove the insoluble material.

NPM mutagenesis

NPM mutants containing threonine to alanine mutations at Thr²³⁴ and Thr²³⁷ were generated using the full-length human NPM cDNA cloned into the *Bam*HI–*Xho*I site of the pCMV3 vector as the template. First, an N-terminal HA (haemagglutinin)-tagged wild-type NPM was generated by subcloning NPM into the *Bam*HI and *Hind*III sites of the pMCL vector containing the HA-tag sequence [17]. The NPM wild-type was amplified by PCR using the following primers: 5'-CGCGGATCCGCGATGGAAG-ATTCGATGGAC-3' (*Bam*HI site is underlined) and 5'-CCC-AAGCTTGGGCTAAAGAGACTTCTCCA-3' (*Hind*III site is

underlined). Next, the Quik Change site-directed mutagenesis kit (Stratagene) was used to generate the double threonine to alanine mutation on residues 234 and 237 using HA-NPM wild-type as the template and the following primers (the codons for alanine are underlined): 5'-CAGGAAAAAGCTCCTAAAGCACCAAA-AGGACC-3' and 5'-GGTCCTTTGGTGCTTTAGGAGCTTT-TTCCTG-3' respectively. The NPM phosphorylation site mutants were verified by DNA sequencing.

Immunofluorescence

Cells were fixed either in 4 % (w/v) paraformaldehyde, followed by permeabilization with 0.1 % Triton X-100, or in cold methanol followed by cold acetone. Both fixation methods gave similar staining results. Immunofluorescence analysis was performed as described previously [4,14]. Staining intensity of phosphorylated NPM using the phospho-MKK1/MKK2 or HA antibodies at the centrosome was quantified from captured digital images using IPLab Scientific Imaging Software (Scanalytics, Fairfax, VA, U.S.A.) for cells transfected with wild-type NPM, NPM phosphorylation site mutants or nocodazole-arrested cells treated in the absence or presence of staurosporine (1 μ M) or roscovitine (60–100 μ M) during the last 1–2 h of incubation. Using IPLab Imaging Software, an ROI (region of interest) was drawn as a circle around the centrosome region, which was verified by co-immunostaining with γ -tubulin. The average pixel intensity and S.D. of the ROI were obtained for control cells and NPM mutant-transfected or inhibitor-treated cells. We identified 15–20 metaphase cells, each containing a pair of centrosomes, and used the average pixel intensity for antibody staining at each pair of chromosomes to calculate the mean and S.D. for each condition.

Immunoprecipitation and kinase assays

In vitro kinase assays consisted of Cdc2 that was immunoprecipitated from cells synchronized in G₁/S- or G₂/M-phase and then added to cell lysates (10 μ l) generated from G₁/S-phase-synchronized cells. The kinase reaction mixture (25 μ l) consisted of 25 mM Hepes (pH 7.4), 15 mM MgCl₂, 1 mM dithiothreitol, 20 μ M unlabelled ATP and 10 μ Ci of [γ -³²P]ATP. In some cases, immunoprecipitated Cdc2 was incubated with His₆-tagged NPM wild-type or GST (glutathione S-transferase)-tagged NPM C-terminal deletion mutants (0.5–1 μ g) that were purified from bacteria, as described in [18]. After incubation for 60 min at 30 °C, the reaction was stopped with SDS/PAGE sample buffer and the kinase reaction mixture was immunoblotted for phospho-MKK1/MKK2, NPM, Cdc2 or α -tubulin as a loading control. Phosphate incorporation into NPM was quantified by Phosphor-Imager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Phosphatase assay

CIP (calf intestinal phosphatase; 10 units) was added to the lysates (approx. 20 μ g of total protein) isolated from G₂/M-phase-synchronized cells in 100 mM NaCl, 50 mM Tris/HCl (pH 7.9), 10 mM MgCl₂ and 1 mM dithiothreitol. The samples were then allowed to incubate for 30 min at 37 °C. The reactions were stopped with SDS/PAGE sample buffer and immunoblotted with the phospho-MKK1/MKK2 antibody.

Protein isolation and MS analysis

The proteins for MS analysis were immunoprecipitated using the phospho-MKK1/MKK2 antibody from protein lysates generated from HeLa cells (10⁸) synchronized in G₂/M-phase. Cells were lysed in 1 ml of TLB and centrifuged at 20 000 g to clarify the lysate. The supernatant was saved; the pellet was re-extracted

with 300 ml of TLB containing 0.1 % SDS, clarified at 20 000 *g* and the resulting supernatant was combined with the first supernatant. The supernatants were then incubated with 10 μ l of phospho-MKK1/MKK2 antibodies (0.1 mg/ml) overnight at 4 °C to immunoprecipitate proteins. The next day, 200 μ l of 50 % (v/v) Protein A–Sepharose in PBS slurry was added to the lysates plus antibody and the lysates were incubated overnight at 4 °C. As a control, the lysates were incubated with Protein A–Sepharose alone in the absence of the phospho-MKK1/MKK2 antibody. The control and immunoprecipitation mixtures were then applied on to a small column and washed with 10 ml of TLB. The co-interacting proteins associated with the control or phospho-MKK1/MKK2 immunoprecipitation were eluted with 100 μ l of 2 $\times$ SDS/PAGE sample buffer.

Half of the eluted proteins (50 μ l) were separated by SDS/PAGE and stained with Colloidal Brilliant Blue G (Sigma), and the gel region corresponding to 35–40 kDa was cut using a sterile razor into five sequential slices (gel thickness, approx. 1 mm $\times$ 7 mm $\times$ 1 mm) numbered 1 to 5. A small portion (approx. 10 %) of each gel slice was then re-loaded into separate wells, subjected to SDS/PAGE and immunoblotted for phospho-MKK1/MKK2. The remainder of each gel slice was washed with sterile double distilled water and air-dried. The gel slices that were positive for phospho-MKK1/MKK2 in the immunoblot were then combined and analysed by MS. As a control, the phospho-MKK1/MKK2-positive gel slices were immunoblotted for actin, which is an abundant protein near this molecular mass and is found to associate non-specifically with the Sepharose beads. Only gel, slices that showed negative reactivity for actin after immunoblotting were used in the MS analysis. Proteins contained in the phospho-MKK1/MKK2-positive gel slices and the corresponding region of the Sepharose only gel, to control for chemical noise or non-specific protein background, were analysed at the Harvard Microchemistry Facility (Harvard University, Cambridge, MA, U.S.A.) by microcapillary reverse-phase HPLC nanoelectrospray tandem MS on a Finnegan LCQ DECA XP quadrupole ion-trap mass spectrometer.

RESULTS

Generation of a smaller-molecular-mass protein related to phosphorylated MKK1/MKK2 during G₂/M-phase

MKK1/MKK2 proteins have been implicated in regulating cell-cycle transitions during G₂/M-phase [1,2]. We have examined MKK1/MKK2 regulation by immunoblotting for active MKK1/MKK2 (pMKK1/MKK2), which is phosphorylated on its activating serine residues in synchronized HeLa cells. Cells were synchronized at the G₁/S boundary using a double thymidine block, released back into the cell cycle and harvested at various times as cells transitioned through S-, G₂- and M-phases. Immunoblotting for pMKK1/MKK2 with a phospho-MKK1/MKK2 antibody revealed an approx. 35 kDa protein that appeared transiently 7 and 9 h after release from G₁/S block, but revealed little or no reactivity with the full-length (approx. 45–46 kDa) MKK1 or MKK2 proteins (Figures 1A and 1B). In agreement with other authors [4,15], 7–9 h after G₁/S release corresponded to the time when HeLa cells were in G₂/M-phase of the cell cycle. It was verified by the observation of high expression levels of cyclin B1 (Figure 1A), flow-cytometry analysis and Cdc2 histone kinase activity assays (results not shown). As a control, preincubation of the phospho-MKK1/MKK2 antibody with the antigenic peptide eliminated reactivity with the 35 kDa protein (Figure 1B). The 35 kDa protein that reacted with the phospho-MKK1/MKK2 antibody was observed in multiple cell lines during G₂/M-phase and

with a phospho-MKK1/MKK2 antibody from another commercial source (results not shown).

Given that MKK1/MKK2 phosphorylation is regulated by growth factor receptors, we tested whether reactivity of the 35 kDa protein with the phospho-MKK1/MKK2 antibody could be induced at other times during the cell cycle. Cells arrested at the G₁/S-phase boundary with excess thymidine were released back into the cell cycle and treated with EGF (epidermal growth factor) for 5 min before harvesting at various time points after release. The protein lysates from untreated and EGF-treated cells were then immunoblotted for pMKK1/MKK2. Although the phosphorylation of full-length MKK1/MKK2 was increased at each time point in response to EGF treatment, the reactivity of the 35 kDa protein was not affected (Figure 1C). However, reactivity of the 35 kDa protein with the phospho-MKK1/MKK2 antibody could be observed in interphase cell lysates after incubation with active Cdc2 immunoprecipitated from G₂/M-phase cell lysates (Figure 1D). Thus the 35 kDa protein that reacts with the phospho-MKK1/MKK2 antibody does not appear to respond to growth factors that are known to activate the ERK pathway and may cycle between an unphosphorylated form during interphase and a Cdc2-regulated phosphorylated form during mitotic transitions.

Other MKK1/MKK2 antibodies were tested for their reactivity with the 35 kDa protein. Immunoblotting with phosphorylation-independent MKK1 or MKK2 antibodies, which were raised against peptide regions corresponding to the serine activation sites as well as the N- or C-termini, showed no apparent reactivity with the 35 kDa protein (Figure 1 and results not shown). Furthermore, a phosphorylation-independent MKK1/MKK2 antibody, which was generated against amino acids 265–276 that are conserved in MKK1/MKK2, readily detected full-length MKK1/MKK2, but failed to react with the 35 kDa protein recognized by the phospho-MKK1/MKK2 antibody (results not shown). The inability to recognize the 35 kDa protein with a variety of phosphorylation-independent MKK1/MKK2 antibodies indicated that the phospho-MKK1/MKK2 antibody cross-reacts with a protein that is not related to MKK1 or MKK2.

Phospho-MKK1/MKK2 antibodies recognize phosphorylated nucleophosmin during G₂/M

MS analysis was used to identify the 35 kDa protein(s) recognized by the phospho-MKK1/MKK2 antibody. Cell lysates from G₂/M-phase-synchronized cells were immunoprecipitated with the phospho-MKK1/MKK2 antibody. After separation of the immunocomplex by SDS/PAGE, the region on the gel corresponding to approx. 35 kDa and containing the protein(s) reactive with the phospho-MKK1/MKK2 antibody was excised. The co-immunoprecipitating proteins were then trypsinized and sequenced by nanoelectrospray tandem MS. Peptide sequence analysis identified three proteins, TPM2 (tropomyosin-2; 32.8 kDa), TMO3 (tropomodulin-3; 39.6 kDa) and NPM (32.6 kDa), but not MKK1 or MKK2 in the immunocomplex (results not shown). Both TPM2 and TMO3 are actin-binding proteins that may be regulated by phosphorylation and are involved in cell division [19–23]. However, neither TPM2 nor TMO3 contains proline-directed phosphorylation sites characteristic of Cdk (cyclin-dependent kinase), which we predict to be involved in the phospho-MKK1/MKK2 antibody reactivity during G₂/M-phase on the basis of Figure 1(C). Previous studies report phosphorylation of NPM during mitotic transitions by Cdc2 (Cdk1) and during centrosome duplication in late G₁-phase by Cdk2 [24,25]. On the basis of the preliminary observations in Figure 1 and the MS results, we examined whether phospho-MKK1/MKK2 antibodies cross-reacted with phosphorylated NPM in mitotic cells.

Figure 1 Detection of a 35 kDa protein during G₂/M-phase of the cell cycle using phospho-MKK1/MKK2 antibodies

(A) Protein lysates were collected from G₁/S-phase-synchronized HeLa cells that were released back into the cell cycle for the times indicated. The top panel shows an immunoblot for phosphorylated MKK1/MKK2 (pMKK1/MKK2) with the upper and lower arrows indicating the positions of the full-length and 35 kDa pMKK1/MKK2 reactive proteins respectively. The middle panel shows an immunoblot that was probed simultaneously for cyclin B1 and ERK2. The band just above the ERK2 signal is the slower migrating phosphorylated form indicative of active ERK [4]. Note that the cyclin B1 and ERK2 blot is a re-probe of the pMKK1/MKK2 blot; thus the band just below the total ERK2 is the 35 kDa protein that reacts with the phospho-MKK1/MKK2 antibody as observed in the top panel. The bottom panel shows the expression of total MKK1 (using a C-terminal-specific MKK1 antibody). (B) Synchronized cell lysates were collected at the times indicated after G₁/S release and immunoblotted with phospho-MKK1/MKK2 antibody in the presence (left panel) or absence (Control) of antigenic peptide. (C) Cells synchronized at the G₁/S boundary were released back into the cell cycle. The cells were treated, 5 min before harvesting at the times indicated, in the absence (controls) or presence of EGF (50 ng/ml). Phosphorylated MKK1/MKK2 (pMKK1/MKK2) and α -tubulin (top and bottom panels respectively) for a protein-loading control were examined by immunoblotting. The upper and lower arrows in the top panel indicate the positions of the full-length MKK1 and the 35 kDa proteins recognized by the phospho-MKK1/MKK2 antibody respectively. (D) Interphase cell extracts were incubated with Cdc2 immunoprecipitated from G₁/S- or G₂/M-phase-synchronized cells. After incubation, the reactions were immunoblotted for pMKK1/MKK2, Cdc2 or α -tubulin for a protein-loading control. Results are representative of three independent experiments.

Cells were first arrested in prometaphase of mitosis with nocodazole and then washed to allow cells to exit from mitosis. Samples were collected at time points after release and cell lysates were immunoblotted with antibodies raised against total NPM and pMKK1/MKK2. The total NPM protein appeared as a doublet in nocodazole-arrested cells, supporting the presence of phosphorylated and non-phosphorylated NPM (Figure 2A). At 1 and 2 h after release from nocodazole, the gel-retarded NPM band was diminished, indicating that NPM was dephosphorylated as cells re-entered the G₁-phase of the cell cycle (Figure 2A). The phospho-MKK1/MKK2 antibody showed strong immunoreactivity with a protein that migrated at the same molecular mass as the gel-retarded NPM in nocodazole-arrested cells (Figure 2A). In addition, phospho-MKK1/MKK2 reactivity at this molecular mass decreased significantly as cells re-entered the G₁-phase (Figure 2A), indicating that phospho-MKK1/MKK2 antibody probably recognized the phosphorylated form of NPM.

Next, nocodazole-arrested cells were incubated in the absence or presence of the kinase inhibitor staurosporine and immunoblotted for total or phosphorylated NPM. Staurosporine inhibited not only the gel-retarded form of NPM recognized by the total NPM antibody but also the phosphorylated protein that co-migrated with gel-retarded NPM and was recognized by the phospho-MKK1/MKK2 antibody (Figure 2B). Similarly, phosphatase treatment of mitotic cells also inhibited the reactivity of the phospho-MKK1/MKK2 antibody with the phosphorylated protein that migrated at the same molecular mass as the mass of the

gel-retarded NPM (Figure 2C). Thus these results strongly suggest that the phospho-MKK1/MKK2 antibody recognizes NPM that is phosphorylated during mitosis.

NPM and phosphorylated NPM localize to similar intracellular regions in mitotic cells

To examine further the ability of the phospho-MKK1/MKK2 antibody to recognize phosphorylated NPM, the cellular localization of NPM and phospho-MKK1/MKK2 was examined by biochemical fractionation and immunofluorescence analysis. First, synchronized cell lysates that were fractionated into soluble and membrane-associated proteins were isolated from either the cytoplasm or the nucleus. The protein that was recognized by the phospho-MKK1/MKK2 antibody and was at the same molecular mass as NPM was found in the membrane protein fractions associated with the cytoplasm or the nucleus, and also with the soluble nuclear protein fraction from cells synchronized in G₂/M-phase (Figure 3A). Total NPM reactivity also localized to these intracellular fractions irrespective of whether the cells were isolated from G₁/S- or G₂/M-phase-synchronized cells (Figure 3A). Total NPM also showed a higher level associated with the cytoplasmic membrane fraction in G₂/M-phase-synchronized cells, which is consistent with its role in regulating centrosome function. As a control, the expression of total MKK1 showed localization primarily to the soluble cytosolic fractions of both G₁/S- and G₂/M-phase-synchronized cells (Figure 3A).

Figure 2 Recognition of phosphorylated NPM during G₂/M-phase using phospho-MKK1/MKK2 antibodies

(A) Cells arrested during mitosis with nocodazole were released from arrest and the lysates were collected after 5, 60 and 120 min. Samples were immunoblotted for NPM or pMKK1/MKK2, followed by re-probing for total MKK1 as a protein-loading control. NPM immunoblots showed two bands in nocodazole-arrested cells with loss of the upper band as cells exited mitosis. The upper phosphorylated-NPM (pNPM) band co-migrated with the protein recognized by the phospho-MKK1/MKK2 antibody. (B) Incubation of nocodazole-arrested cells with the kinase inhibitor staurosporine (ST; 1 μ M) for 1 h before harvesting inhibited the slower migrating form of NPM and pMKK1/MKK2 immunoreactivity. (C) Cell lysates generated from G₂/M-phase-synchronized cells were incubated in the absence or presence of CIP. Phosphatase treatment of cell lysates inhibited pMKK1/MKK2 reactivity in G₂/M-phase lysates.

Since phosphorylated NPM appears to be the major protein recognized by the phospho-MKK1/MKK2 antibody in mitotic cells as determined by immunoblotting, we next examined NPM and phospho-MKK1/MKK2 localization in mitotic cells by immunofluorescence analysis. Staining for total NPM showed cytoplasmic and strong nucleolar staining (Figure 3B). Phospho-MKK1/MKK2 staining also revealed nucleolar staining in a select population of cells that were in early prophase, as suggested by the nuclear accumulation of cyclin B1 and chromosome condensation (Figure 3B). These results indicate that NPM in the nucleolus was phosphorylated before the breakdown of nuclear envelope. In contrast, NPM staining was not observed at the centrosomes of these cells identified to be in early mitosis (Figure 3B). However, after the breakdown of nuclear envelope, total NPM and phosphorylated NPM recognized by the phospho-MKK1/MKK2 antibody localized to the centrosome in metaphase cells (Figure 3C), which is in agreement with previous reports [3,25]. This staining pattern was observed when either 4% paraformaldehyde or cold methanol/acetone was used during the fixation procedure (results not shown). These results provide evidence that phos-

phorylated NPM is targeted to the centrosome during mitosis and that the phospho-MKK1/MKK2 antibody is a useful tool to monitor NPM regulation and function during G₂/M-phase of the cell cycle.

Phospho-MKK1/MKK2 antibodies recognize NPM phosphorylated by Cdc2

Cdc2 phosphorylates NPM on at least two threonine residues at positions 234 and 237 during mitosis. However, the functional role of these phosphorylations is not known [24,26]. In contrast, phosphorylation of threonine at position 199 by Cdk2 during late G₁-phase is reported to cause NPM dissociation from the centrosome and is required for proper centrosome duplication [26,27]. Using purified NPM as a substrate, we next determined whether the phospho-MKK1/MKK2 antibody recognized NPM that was phosphorylated at the C-terminus by Cdc2. Cdc2 was immunoprecipitated from lysates generated from G₁/S- or G₂/M-phase-synchronized cells and incubated with purified NPM protein in the presence of radioactive ATP. Incorporation of radiolabelled phosphate into NPM was highest after incubation with G₂/M-phase Cdc2 (Figure 4A). Similarly, the phospho-MKK1/MKK2 antibody only detected NPM that was phosphorylated by Cdc2 immunoprecipitated from G₂/M-phase cell lysates (Figure 4B). As predicted, an NPM deletion mutant lacking the C-terminal Cdc2 phosphorylation sites was not phosphorylated by active Cdc2 (results not shown) or detected by the phospho-MKK1/MKK2 antibody (Figure 4C), supporting the idea that the Cdc2 phosphorylation sites are required for antibody recognition.

Furthermore, we localized the site on NPM that is recognized by the phospho-MKK1/MKK2 antibody using additional C-terminal NPM deletion mutants. GST-tagged NPM proteins containing C-terminal amino acids 175–222 or 225–293 were incubated with Cdc2 immunoprecipitated from mitotic cell lysates and then immunoblotted for phosphorylated NPM using the phospho-MKK1/MKK2 antibody. Phospho-MKK1/MKK2 antibody reactivity was observed with the GST–NPM (225–293) mutant that contains the Cdc2 phosphorylation sites, but not with the GST–NPM (175–222) mutant (Figure 4D). Since the GST–NPM (225–293) mutant contains the proline-directed Cdc2 phosphorylation sites on threonine residues at 234 and 237, these results suggest that the phospho-MKK1/MKK2 antibody reactivity with NPM is dependent on one or both of these Cdc2 phosphorylation sites during mitosis. This was verified by generating threonine to alanine mutations T^{234,237}A (Thr^{234,237} → Ala) at Thr²³⁴ and Thr²³⁷ of NPM. Cells expressing wild-type NPM or the T^{234,237}A mutant were arrested in mitosis by nocodazole treatment. The expressed NPM proteins were immunoprecipitated using an HA tag and immunoblotted for HA or phospho-MKK1/MKK2. As predicted, no reactivity with the phospho-MKK1/MKK2 antibody was observed with the NPM T^{234,237}A phosphorylation site mutant, although reactivity was readily seen with wild-type NPM (Figure 4E).

Phosphorylation regulates NPM targeting to the centrosome during mitosis

Although phosphorylation appears to regulate NPM dissociation from the centrosome during the late G₁- and the early S-phase, the mechanisms of regulating NPM re-association with the centrosome during mitosis have not been examined. Using the phospho-MKK1/MKK2 antibody as a marker of phosphorylated NPM, we examined the requirement for phosphorylation in targeting NPM to the centrosome during mitosis. Cells arrested in mitosis with nocodazole were treated in the absence or presence of the general

Figure 3 Co-localization of NPM and phospho-MKK1/MKK2 antibody reactivity in G₂/M-phase cells

(A) Cells synchronized in G₁/S- or G₂/M-phase were separated into cytoplasmic (Cyto.) or nuclear (Nuc.) protein fractions. The protein fractions were further separated into soluble (C) or total membrane (M) proteins by ultracentrifugation and immunoblotted with phospho-MKK1/MKK2 (top panel), total NPM (middle panel) or total MKK1 (bottom panel) antibodies. Results are representative of three separate experiments. (B, C) For immunofluorescence, HeLa cells were fixed in cold methanol and co-immunostained for cyclin B1 and total NPM or phosphorylated NPM using the phospho-MKK1/MKK2 antibody. Mitotic cells (indicated by arrows) were identified by nuclear cyclin B1 and by counterstaining with DAPI to identify condensed mitotic chromosomes. NPM and phospho-MKK1/MKK2 immunoreactivity showed similar localization to nucleoli and centrosomes during (B) prophase or (C) metaphase respectively. The cell-staining results were obtained from multiple mitotic cells for three separate experiments.

kinase inhibitor staurosporine or the Cdk-specific inhibitor roscovitine during the last hour of nocodazole treatment. As shown in Figure 5(A), staurosporine and roscovitine blocked NPM phosphorylation as well as Cdc2 histone kinase activity (Figure 5B). Next, these inhibitors were used to test the requirement for phosphorylation in targeting NPM to the centrosome in mitotic cells. Both staurosporine and roscovitine caused significant inhibition of phosphorylated NPM that associated with the centrosome during the metaphase of mitosis, as determined by immunofluo-

rescence analysis using the phospho-MKK1/MKK2 antibody (Figures 6A and 6B). These findings indicate that re-association of NPM at the centrosome during mitosis is regulated by Cdc2-mediated phosphorylation.

To demonstrate further the requirement for mitotic phosphorylation by Cdc2 at Thr²³⁴ and Thr²³⁷ in regulating NPM centrosome localization, HeLa cells overexpressing the NPM (T^{234,237}A) phosphorylation site mutant and wild-type protein were examined by immunofluorescence analysis during mitosis. Mitotic cells in

Figure 4 Cross-reactivity of the phospho-MKK1/MKK2 antibody with NPM phosphorylated by active Cdc2 at Thr²³⁴ and Thr²³⁷

(A) Cdc2 was immunoprecipitated from cells synchronized in G₂/M- or G₁/S-phase of the cell cycle and incubated with 0.5 µg of bacterially purified NPM as a substrate in an *in vitro* kinase assay. Radiolabelled phosphate incorporation (PI) into NPM and its quantification by PhosphorImager analysis are shown in the upper and lower panels respectively. (B) Phospho-MKK1/MKK2, Cdc2 and NPM immunoblots from samples listed in (A) are shown in the top, middle and bottom panels respectively. Non-specific kinase activity is shown, using Protein G-Sepharose (beads) only as a control. (C) Wild-type NPM and a GST-tagged NPM mutant containing amino acids 1–180 were incubated with Cdc2 immunoprecipitated from G₁/S- or G₂/M-phase lysates in a kinase assay. The kinase reaction was resolved by SDS/PAGE and immunoblotted using a total NPM antibody (left panel) and the phospho-MKK1/MKK2 antibody (right panel) as a marker of phosphorylated NPM. Results are representative of three independent experiments. (D) GST-tagged NPM mutants containing amino acids 175–222 or 225–293 were incubated with Cdc2 immunoprecipitated from G₂/M-phase lysates in an *in vitro* kinase assay. Phosphate incorporation into NPM (pNPM) was measured by immunoblotting with the phospho-MKK1/MKK2 antibody (top panel) and PhosphorImager analysis (bottom panel). A GST immunoblot shows the relative amounts of the NPM mutants under each condition (middle panel). Results are representative of two separate experiments. (E) Wild-type NPM or the T^{234,237}A mutant were expressed in cells, which were then treated with nocodazole for 14 h to arrest cells in mitosis. The NPM proteins were immunoprecipitated with an HA antibody and the immunoprecipitates were immunoblotted using the HA (top panel) or phospho-MKK1/MKK2 (pMKK1/MKK2) antibody (middle panel). Arrows indicate the location of the expressed NPM proteins. Expression of wild-type and mutant HA–NPM in the total lysates is shown in the bottom panel.

metaphase that expressed mutant or wild-type NPM were identified by immunostaining for the HA tag on the NPM proteins and DAPI (4,6-diamidino-2-phenylindole) staining of chromosomes (Figure 7A). As shown, centrosome staining of wild-type NPM was apparent in cells stained with the HA antibody, whereas HA staining at the centrosome of the expressed NPM (T^{234,237}A) mutant was markedly inhibited (Figure 7A). HA antibody-staining intensity at the centrosome, which was quantified as in Figure 6(B), indicated a more than 2.5-fold decrease in the NPM (T^{234,237}A) mutant that associated with the centrosome when compared with wild-type protein (Figure 7B). These results support the requirement for Cdc2-mediated phosphorylation of Thr²³⁴ and Thr²³⁷ in targeting NPM to the centrosome (Figure 7B).

DISCUSSION

Using MS analysis, the present study identifies phosphorylated NPM as a protein that cross-reacts with phospho-MKK1/MKK2 antibodies during G₂/M-phase of the cell cycle. Furthermore, we demonstrate that phospho-MKK1/MKK2 antibody cross-reactivity

is dependent on NPM that is phosphorylated on threonine residues at 234 and 237, which are targeted by Cdc2 during mitosis. This antibody cross-reactivity is somewhat surprising given that the peptide sequence LIDSMANSFVGTR (regulatory phosphorylation sites are underlined), which was used to make the phospho-MKK1/MKK2 antibody, shows very little sequence similarity to the region around the known Cdc2 phosphorylation sites (Thr²³⁴ and Thr²³⁷) on human NPM (primary accession no. P06748). Nonetheless, given that little is known about the role of phosphorylation in regulating NPM function during mitosis, these findings indicate that commercially available phospho-MKK1/MKK2 antibodies can be useful tools for studying NPM regulation during mitotic transitions. As such, we demonstrate that phosphorylation of the 234 and 237 sites is required for recognition by the phospho-MKK1/MKK2 antibody (Figure 4) and is important for targeting NPM to the centrosome in mitotic cells (Figures 6 and 7).

A recent study suggested the interesting possibility that the 35 kDa protein recognized by the phospho-MKK1/MKK2 antibodies during mitosis was a proteolytic product of phosphorylated MKK1 and that this partial proteolysis functioned to

Figure 5 Prevention of generation of phosphorylated NPM during the G₂/M-phase by kinase inhibitors

Cells were synchronized in G₁/S-phase with excess thymidine and released back into the cell cycle. Cells were incubated, 8 h after G₁/S release, for an additional 1 h with staurosporine (ST; 1 μ M) or the Cdk/Cdc2 inhibitor roscovitine (RC; 50 μ M) and the cell lysates were then collected. **(A)** Immunoblotting of cell lysates for phosphorylated NPM (pNPM) using the phospho-MKK1/MKK2 antibody or α -tubulin as a protein-loading control. **(B)** Relative histone kinase activity in control, staurosporine- or roscovitine-treated cells after immunoprecipitation of Cdc2 from G₂/M cell lysates. Results are representative of two separate experiments.

Figure 6 Dependence on Cdc2 kinase activity for NPM localization to the centrosome during mitosis

Cells were treated in the absence (control) or presence of staurosporine (ST; 1 μ M) or roscovitine (RC; 100 μ M) for 2 h. After fixation, phosphorylated NPM (pNPM) staining at the centrosome of metaphase cells was examined using the phospho-MKK1/MKK2 antibody. **(A)** Representative images of pNPM staining in control cells and staurosporine- or roscovitine-treated cells are shown. DAPI staining of chromosomes is shown to identify mitotic cells in metaphase. **(B)** The relative amount of pNPM at the centrosome was quantified by taking the average pixel intensity of pNPM staining from digital images of control, staurosporine- or roscovitine-treated cells. Results are the means $\pm$ S.E.M. for pNPM staining values obtained from 15 cells for each condition.

Figure 7 Dependence on the phosphorylation of Thr²³⁴ and Thr²³⁷ for NPM localization to the centrosome during mitosis

HeLa cells were transfected with wild-type NPM (WT) and the T^{234,237}A (AA) phosphorylation site mutant, and metaphase cells were identified by DAPI staining. **(A)** Immunofluorescence analysis of HA-tagged wild-type NPM (top panels) and the T^{234,237}A mutant (bottom panels) in metaphase cells using the HA antibody. Arrows indicate the position of the centrosome. The corresponding DAPI staining of chromosomes for each condition is shown. **(B)** Quantification of the relative amounts of wild-type HA-NPM (WT) or T^{234,237}A mutant (AA) at the centrosome was performed as described in Figure 6. Results are the means $\pm$ S.E.M. for the centrosome pair of seven separate metaphase cells under each condition and were reproduced in two separate experiments.

uncouple MKK1 from signalling through the ERK MAP kinases [10]. Although intriguing, evidence that MKK1 proteolysis actually occurs or serves a functional role during mitosis is lacking. For example, total MKK1 or MKK2 protein levels do not change significantly throughout the cell cycle, which indicates that the proteolysed form of MKK1, if it existed, only represents a very small fraction of the total MKK1 protein in the cell and is not capable of coupling with ERK activation. Furthermore, previous studies did not confirm that the proteolysed protein was indeed related to MKK1 using methods other than immunoblotting [10]. In contrast, our results and the discrepancies of previous studies provide strong evidence that active MKK1 does not undergo specific proteolysis during mitosis.

Although NPM exists as a phosphoprotein, little is known about how specific phosphorylation sites regulate NPM function. The best-described example is for Cdk2 phosphorylation of NPM at Thr¹⁹⁹ during late G₁- and early S-phases. Under these conditions, phosphorylated NPM dissociates from the centrosome, which appears to be necessary for centrosome duplication to occur [26,27]. In contrast, the function of phosphorylated NPM during mitotic transitions has not been examined. Although one study reported that inhibition of NPM protein levels using antisense mRNA caused a delay in mitotic entry and suggested that NPM is required for regulating the structural changes necessary for

proper mitotic entry [28], the role of phosphorylation events was not examined. In agreement with the results of a previous study [25], our results support a model where NPM is targeted to the centrosome after it is phosphorylated by Cdc2 and after mitotic nuclear envelope breakdown. The specific role for phosphorylated NPM in regulating centrosome function during mitosis awaits further study. However, as one possibility, phosphorylated NPM may regulate the assembly or targeting of centrosome proteins required for mitotic microtubule formation. This is supported by the role that phosphorylation events play in regulating NPM function in shuttling proteins from one intracellular location to another [29]. For example, NPM phosphorylation at Ser-125 by casein kinase II (CK2) inhibits the ability of NPM to interact with substrates and perform its function as a molecular chaperone [11]. Whether NPM phosphorylated during mitosis functions to shuttle proteins to the centrosome during mitosis is yet to be determined. Regardless, the function of Cdc2-phosphorylated NPM regulation of the mitotic centrosome appears to be distinct from centrosome-bound NPM that is phosphorylated by Cdk2 and released from the duplicating centrosome during late G₁- and early S-phases.

In summary, our findings demonstrate that commercially available phospho-MKK1/MKK2 antibodies cross-react with Cdc2-phosphorylated NPM during mitotic transitions and can be used to monitor NPM regulation and function at this time of the cell cycle. However, conclusions on MKK1/MKK2 regulation and function during mitotic transitions using phospho-MKK1/MKK2 antibodies should be approached with caution.

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