Active MAP Kinase in Mitosis: Localization at Kinetochores and Association with the Motor Protein CENP-E

Maja Zecevic,* Andrew D. Catling,* Scott T. Eblen,* Luigina Renzi,‡ James C. Hittle,§ Tim J. Yen,§ Gary J. Gorbsky,‡ and Michael J. Weber*

*Department of Microbiology and Cancer Center and ‡Department of Cell Biology, University of Virginia, Health Sciences Center, Charlottesville, Virginia 22908; and §The Fox Chase Cancer Institute, Philadelphia, Pennsylvania 19111

Abstract. To investigate possible involvement of the mitogen-activated protein (MAP) kinases ERK1 and ERK2 (extracellular signal-regulated kinases) in somatic cell mitosis, we have used indirect immunofluorescence with a highly specific phospho-MAP kinase antibody and found that a portion of the active MAP kinase is localized at kinetochores, asters, and the midbody during mitosis. Although the aster labeling was constant from the time of nuclear envelope breakdown, the kinetochore labeling first appeared at early prometaphase, started to fade during chromosome congression, and then disappeared at midanaphase. At telophase, active MAP kinase localized at the midbody. Based on colocalization and the presence of a MAP kinase consensus phosphorylation site, we identified the kinetochore motor protein CENP-E as a candidate mitotic substrate for MAP kinase. CENP-E was phosphorylated in vitro by MAP kinase on sites that are known to regulate its interactions with microtubules and was found to associate in vivo preferentially with the active MAP kinase during mitosis. Therefore, the presence of active MAP kinase at specific mitotic structures and its interaction with CENP-E suggest that MAP kinase could play a role in mitosis at least in part by altering the ability of CENP-E to mediate interactions between chromosomes and microtubules.

Key words: MAP kinase • CENP-E • kinetochore • mitosis • phosphorylation

M ITOGEN-ACTIVATED protein kinases (MAP kinases)[1] or extracellular signal–regulated kinases (ERKs) are serine/threonine-specific protein kinases (Ray and Sturgill, 1987; Rossomando et al., 1989; Boulton et al., 1991) that are activated in response to extracellular signals and play important roles as effectors for diverse cellular functions, including growth, differentiation, movement, and secretion (L'Allemain, 1994). MAP kinases are activated by dual threonine and tyrosine phosphorylation on a TEY sequence located in the regulatory lip and these phosphorylations are both necessary and sufficient for enzymatic activation (Anderson et al., 1990; Zhang et al., 1995). Both phosphorylations are catalyzed by MEKs (MAPK/ERK Kinases), which are dual-specificity MAP kinase kinases (Crews et al., 1992; Nakielny et al., 1992; Her et al., 1993; Zheng and Guan, 1993). MEKs, in turn, are activated by phosphorylation on serine residues by various serine/threonine protein kinases, especially members of the Raf family (Dent et al., 1992; Huang et al., 1993; Alessi et al., 1994; Zheng and Guan, 1994).

The best-studied function of MAP kinases has been in the regulation of the $G_o \rightarrow G_1 \rightarrow S$ transition (Kahan et al., 1992; Meloche et al., 1992; Pages et al., 1993). After activation in response to extracellular agonists, MAP kinases translocate to sites of action, including the nucleus (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993). Phosphorylation of transcription factors and concomitant regulation of gene expression by MAP kinases is a key early event in initiating cell cycle progression by growth factors. The localization of activated MAP kinase at its nuclear site of action is believed to be essential for these events.

Another well-established role of MAP kinase is in the

regulation of meiosis in *Xenopus*, where it functions as an effector for Mos, a germline-specific serine/threonine kinase. Mos initiates oocyte maturation in meiosis I by activating the maturation-promoting factor (MPF) and functions in metaphase II as a component of the cytostatic factor (CSF) in unfertilized eggs. Mos is also essential for CSF function in mouse oocytes (Vande Woude, 1994). Mos can activate MEK in vitro (Nebreda et al., 1993; Posada et al., 1993) and is therefore considered, like Raf, to be able to function as a MAP kinase kinase kinase. In *Xenopus*, oocyte maturation in the presence of Mos was inhibited when MAP kinase activation was prevented by injection of anti-MEK neutralizing antibodies (Kosako et al., 1994*b*) or MKP-1, a MAP kinase phosphatase (Gotoh et al., 1995). Conversely, the injection of active thiophosphorylated MAP kinase (Haccard et al., 1995) or mRNA of constitutively activated MEK (Gotoh et al., 1995) mimicked the effects of Mos with respect to oocyte maturation. In the case of *Xenopus* early embryos, microinjection of thiophosphorylated MAP kinase induced a metaphase arrest resembling the CSF arrest induced by Mos (Haccard et al., 1993), whereas injection of anti-MEK neutralizing antibody prevented the Mos-induced arrest (Kosako et al., 1994*a*). Thus, it is clear that MPF activation by Mos and the CSF activity of Mos are mediated by MAP kinase.

Substantial evidence indicates that MAP kinase also plays a role in the spindle checkpoint in *Xenopus* egg extracts. This checkpoint is activated by chromosome misalignment or a defective spindle and prevents entrance into anaphase. *Xenopus* egg extracts supplemented with sperm nuclei and treated with nocodazole were found to arrest in a mitotic state with high cyclin-dependent kinase 2 (cdc2)/cyclin B and MAP kinase activity (Minshull et al., 1994). Addition of the MAP kinase phosphatase MKP-1 into the M phase–arrested extracts resulted in MAP kinase inactivation, cyclin B destruction, and exit from the arrest. If MKP-1 phosphatase addition preceded nocodazole treatment, the extracts cycled without arrest. Similarly, the arrest did not occur in egg extracts from which MAP kinase had been immunodepleted (Takenaka et al., 1997). Therefore, active MAP kinase is required for both the establishment and maintenance of the M phase arrest induced by spindle depolymerization in these egg extracts. Likewise, maintenance of elevated MAP kinase activity after fertilization blocks cell cycle progression at several points in the first postfertilization cell cycle, including repression of DNA synthesis (Tachibana et al., 1997), prevention of the G_2 to M phase transition, or blockage of M phase progression (Abrieu et al., 1997; Walter et al., 1997; Bitangcol et al., 1998).

Although MAP kinase has established roles in germline meiosis and in the spindle checkpoint in frog egg extracts, its functions in somatic cell mitosis are less clear. When injected into mitotic cultured PtK1 cells, Mos became associated with kinetochores, induced an abnormal spindle morphology, and blocked the formation of the metaphase plate (Wang et al., 1994). Similarly, other candidate activators of the MAP kinase cascade, such as Ras and Src, have been reported to induce genomic instability and abnormal mitosis (Hagag et al., 1990; Denko et al., 1994) or are required for M phase entrance (Roche et al., 1995), respectively. However, it is not known whether these effects are

mediated by MAP kinase. The most direct approach to examine the relation between MAP kinase and somatic cell division was the report of Wang et al., 1997. These workers showed that XTC cells microinjected with the MAP kinase phosphatase XCL100 did not display the characteristic mitotic block when treated with nocodazole. This is consistent with the hypothesis that sustained MAP kinase activity might be required for the spindle checkpoint in somatic cells, as it is in cycling egg extracts. However, recent work suggests that these functions could be ascribed to the p38 stress-activated protein kinase, which is a MAP kinase homologue (Takenaka et al., 1998).

Here we present evidence that supports the involvement of MAP kinase in normal somatic cell mitosis. Active MAP kinase was localized at kinetochores, asters, and the midbody of dividing PtK1 cells, using a phospho-MAP kinase antibody that is specific for the dually phosphorylated and activated MAP kinases (ERK1 and ERK2). The activated MAP kinase appeared on kinetochores during early prometaphase and became undetectable by midanaphase. Thus, the active MAP kinase was located at a site where it could play a role in regulation of mitotic progression, and the time course of its appearance and disappearance was consistent with a role in the regulation of chromosome movement. We also show that MAP kinase is able to phosphorylate the kinetochore motor protein CENP-E on sites that regulate the interaction of centromere-binding protein E (CENP-E) with microtubules. Finally, we demonstrate that MAP kinase and CENP-E can be coimmunoprecipitated, with CENP-E interacting preferentially with inactive MAP kinase during interphase and with phosphorylated MAP kinase during mitosis. Taken together, the results raise the possibility that MAP kinase could play a role in mitotic progression, at least in part, by altering the ability of CENP-E to mediate the interactions between chromosomes and microtubules.

Materials and Methods

Cell Culture

PtK1, NIH3T3, HeLa, and HeLa S3 cells were obtained from the American Type Culture Collection (Rockville, MD). PtK1 and HeLa S3 cells were grown as previously described (Renzi et al., 1997). In some experiments, the mitotic yield of PtK1 cells was increased by a 3–5-h treatment with 5 µg/ml of vinblastine (Sigma Chemical Co., St. Louis, MO). NIH3T3 cells and CCL39 hamster fibroblast were maintained at 37°C and 7.5% CO_2 in DME containing 10% FCS. HeLa cells were grown at 37°C and 5% CO_2 in Iscove's media containing 10% FCS.

To induce quiescence, NIH3T3 and CCL39 cells were grown in 10% FCS DME to 50% confluency, after which they were starved in DME containing 0.2% FCS for 25 h. For stimulation, 20% FCS was added for 15 or 5 min, respectively. PtK1 cells were grown to 80% confluency in 10% FCS MEM and then, without serum deprivation, were stimulated for 15 min with EGF (0.2 µg/ml).

For the CENP-E and MAP kinase coimmunoprecipitation experiments, HeLa cells were grown to 60% confluency. The cells were arrested at mitosis by a 15–17-h treatment with 0.03 µg/ml of nocodazole (Sigma Chemical Co.).

Antibodies

The rabbit phospho-MAP kinase antibody was raised against the phosphopeptide CHTGFLpTEpYVATR. The peptide was coupled to keyhole limpet hemocyanin (KLH) and injected into rabbits to raise polyclonal antisera (Quality Controlled Biochemicals, Inc., Hopkinton, MA). The poly-

clonal antibodies were negatively selected by repeated passage over a column of nonphosphorylated CHTGFL<u>TEY</u>VATR peptide coupled to KLH, and then positively selected by passage over a column of the phosphopeptide coupled to BSA. A concentration of 8.5 μg/ml was used for immunofluorescence and 1.7 μg/ml for Western blotting.

For ERK2-specific recognition by Western blotting, the monoclonal ERK2 antibody 05-157 (Upstate Biotechnology Inc., Lake Placid, NY) was used at a concentration of 0.1 μg/ml, at which it recognizes phosphorylated and unphosphorylated p42 ERK2. For hemagglutinin (HA)-tagged ERK2 immunoprecipitations, 18 μg of monoclonal anti-HA antibody 12CA5 (Berkeley Antibody Co., Richmond, CA) was used per immunoprecipitation. The human CENP-E protein was immunoprecipitated with the affinity-purified rabbit antibody 6A (9 μg/ml) that was generated against the central portion of CENP-E (amino acids 1,250–1,558), whereas Western blotting was performed with the same antibody at a concentration of 0.54 μg/ml. MAP kinase immunoprecipitations from HeLa cells were done with the polyclonal rabbit antibody TR10, made against recombinant ERK2 conjugated to KLH.

All the secondary fluorophore-conjugated antibodies used in indirect immunofluorescence were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The Texas red–conjugated goat anti–rabbit antibody and the fluorescein (FITC)-conjugated goat anti–human antibody were used at a concentration of 7.5 μg/ml. The human autoimmune CREST (calcinosis/Raynaud's phenomenon/esophageal dysmotility/sclerodactyly/telangiectasia variant of scleroderma) scleroderma serum was a gift of J.B. Rattner (University of Calgary, Alberta, Canada).

Preparation of Cell Lysates

For whole cell lysis experiments, PtK1 and NIH3T3 cells were grown to 80% confluency and then lysed in Laemmli sample buffer. The cell lysate was then sonicated, clarified by centrifugation, protein concentration determined, and then 100 μg of protein was used for SDS-PAGE.

CCL39 fibroblasts, stably transfected with HA-tagged wild type ERK2 or ERK2 phosphorylation site mutants (Her et al., 1993), were washed twice in ice-cold PBS and then lysed in cold lysis buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% NP-40, 1 μM pepstatin, 1 μg/ml leupeptin, 1 mM PMSF, 0.2 mM Na vanadate, 2 μg/ml aprotinin, 40 mM 4-nitrophenyl phosphate-disodium salt [PNPP], 50 mM β-glycero-phosphate, 2 mM DTT). Samples were centrifuged at 14,000 rpm for 15 min in a microfuge and protein concentration was estimated by Coomassie dye labeling (Bio-Rad Laboratories, Hercules, CA) before immunoprecipitation.

For CENP-E immunoprecipitations, cells were washed twice in ice-cold PBS and lysed in cold hypotonic buffer (20 mM Hepes, pH 7.4, 2 mM EGTA, 2 mM MgCl$_2$ plus above mentioned inhibitors; used in Fukuda et al., 1997) and centrifuged for 20 min at 14,000 rpm.

Immunoprecipitation

Monoclonal anti-HA antibody 12CA5, affinity-purified rabbit anti-CENP-E 6A antibody or rabbit TR10 antibody were preabsorbed for 1 h at 4°C to protein A–agarose (Boehringer Mannheim Corp., Indianapolis, IN) before incubation with 500 μg of CCL39 cell extract for 4 h, 300 or 800 μg of HeLa cell extract for 3 h at 4°C, respectively. CENP-E immunoprecipitates were transferred to Wizard minicolumns and washed on a vacuum manifold (Promega Corp., Madison, WI). Immunoprecipitates were washed four times with lysis buffer before Laemmli sample buffer was added. The samples were resolved by SDS-PAGE and transferred to nitrocellulose for blotting.

Immunoblotting

All blotting was performed using nitrocellulose (Protran; Schleicher & Schuell, Dassel, Germany). The blocking and primary antibody steps were done at 37°C for 1 h in 5% dry milk in PBS/0.1% Tween 20. The secondary HRP-conjugated anti-mouse and anti-rabbit antibodies or protein A–HRP were incubated for 1 h at room temperature in PBS/0.1% Tween 20. Subsequently, enhanced chemiluminescence was performed using the Amersham Life Science kit (Arlington Heights, IL). Membranes were stripped with stripping buffer (62.5 mM Tris, pH 6.8, 2% SDS, 1% β-mercaptoethanol) for 10 min at 65°C before reprobing.

Cell Fixation and Immunofluorescence

NIH3T3 and PtK1 cells (see Fig. 1, C and D) were stimulated with FCS or EGF, rinsed once with PBS, and then fixed in 0.2% acrolein (Polyscience, Inc., Warrington, PA), 4% formaldehyde, 0.2% Triton X-100, and 2 mM Na vanadate in PBS for 20 min at room temperature. Cells were then quenched with 0.025% NaBH$_4$, 2% glycine in PBS twice for 5 min, and then once for 10 min. Cells were then washed twice with PBS/0.05% Tween 20.

PtK1 cells in Figs. 3, 4, and 5 were first rinsed with PHEM (60 mM Pipes, 25 mM Hepes, pH 6.9, 10 mM EGTA, 4 mM MgSO$_4$) and extracted for 5 min at room temperature with PHEM plus 1% CHAPS and 1 μM pepstatin, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM β-glycero-phosphate and 0.2 mM Na vanadate. Cells were then fixed in 1% formaldehyde in PHEM for 15 min. Cells were rinsed twice with MBST (10 mM MOPS, 150 mM NaCl, 0.05% Tween 20, pH 7.4).

Cells for immunofluorescence were blocked with 20% boiled normal goat serum (NGS; Sigma Chemical Co.) in MBST whereas the primary and secondary antibodies were diluted in MBST containing 5% boiled NGS. Cells were washed with PBS/0.05% Tween 20 for acrolein-fixed cells and MBST for extracted and formaldehyde-fixed cells. All incubations were done by rocking at room temperature for 1 h. After the last wash, cells were counterstained with the DNA dye 4′,6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) at 0.5 μg/ml and were mounted on slides with Vectashield mounting media (Vector Laboratories, Burlingame, CA) containing 10 mM MgSO$_4$. Slides were viewed with a Leitz DMRBE microscope and pictures were taken under the same conditions with the Leitz Vario Orthomat camera (Leica Ltd., Wetzlar, Germany). Subsequently, scanned images were processed using Adobe Photoshop and Illustrator software (Adobe Systems, San Jose, CA).

For the peptide-blocking experiments, the peptides (in an excess of 10-fold mol/mol over antibodies) were preincubated with phospho-MAP kinase antibody and CREST serum for 1 h at room temperature in PBS. After the incubation, the antibodies were applied to extracted and fixed cells that were previously blocked in 20% NGS in MBST.

Chromosome Isolation and Immunofluorescence

Chromosomes were prepared from a 500-ml HeLa S3 culture containing ~5 × 105 cells/ml as previously described (Renzi et al., 1997), except that the glycerol gradient step was omitted and the final chromosome pellet was resuspended in 30 ml of the extraction/lysis buffer.

The isolated chromosomes were pelleted onto precoated (1 mg/ml poly-L-lysine in water for 30 min) 18-mm-round coverslips (Fisher Scientific Co., Pittsburgh, PA) by centrifugation at 1,200 g for 5 min and then fixed with 1% formaldehyde in PHEM. Immunofluorescence was then performed as described above.

In Vitro Phosphorylation Assay

p42 ERK2 was expressed as a soluble nonfusion protein in *Escherichia coli* and purified as described (Wu et al., 1991). It was then in vitro phosphorylated and activated by constitutively active recombinant MEK1 enzyme. The specific activity of MAP kinase was estimated to be 1.2 μmol/min per milligram, using myelin basic protein as a substrate. The COOH-terminal portion (amino acids 2,295–2,663) of human CENP-E proteins (wild-type and mutant) were made from bacteria as a glutathione-S-transferase (GST) fusion proteins. The CENP-E purification and removal of GST was done as previously described (Liao et al., 1994). The CENP-E mutant (CENP-E4A), created by site-directed mutagenesis, has alanines in the place of serines 2,567, 2,570, 2,601, and 2,616. The in vitro phosphorylation of 1.5 μg of the COOH-terminal CENP-E protein (~45 kD) by 160 ng of active MAP kinase was performed at 30°C for 15 min in a final volume of 40 μl containing 25 mM Hepes, pH 7.5, 10 mM Mg (CH$_3$COO)$_2$, 1 mM dithiothreitol, and 0.02 mM [γ-^{32}P]ATP (~22,500 cpm/pmol). Reactions were terminated with sample buffer, products resolved by SDS-PAGE, transferred to nitrocellulose, and then exposed to film.

Results

Phospho-MAP Kinase Antibody Specifically Recognizes the Dually Phosphorylated Form of the ERKs

To determine the cellular localization of activated MAP kinase during mitosis, it was necessary to generate an antibody that was highly specific for the dually phosphorylated, active form of the enzyme and that could be used in

immunocytochemistry as a surrogate for enzyme assays. The phosphopeptide CHTGFLpTEpYVATR corresponding to the region in MAP kinase containing the activating phosphothreonine and phosphotyrosine residues was used as an antigen for antibody production in rabbits and for the subsequent antibody affinity purification, as described in Methods. This sequence is identical in both ERK1 and ERK2 and is conserved in vertebrates.

The specificity of the phospho-MAP kinase antibody was first determined by Western blotting whole cell lysates of NIH3T3 fibroblasts and of PtK1 rat kangaroo epithelial cells, which were used in the immunofluorescence studies described below. When cells were stimulated with FCS (Fig. 1 A) or EGF (Fig. 1 B), the only proteins that reacted with the phospho-MAP kinase antibody corresponded to the phosphorylated forms of the MAP kinases p44 ERK1 and p42 ERK2. However, in serum-deprived cells, only low basal levels of phospho-ERK1 and phospho-ERK2 could be detected using the phospho-MAP kinase antibody.

Reprobing the same blot with a monoclonal antibody against ERK2 demonstrated that the upper band here corresponded to the phosphorylated, activated, and mobility-shifted ERK2 that comigrated with the ERK2 band detected with the phospho-MAP kinase antibody. The ERK2 blot also showed that substantial amounts of the protein remained unphosphorylated and was not recognized by the phospho-MAP kinase antibody.

The phospho-MAP kinase antibody was also used to analyze MAP kinase activation by indirect immunofluorescence. NIH3T3 cells (Fig. 1 C) or PtK1 cells (Fig. 1 D) were stimulated with either serum or EGF, respectively. The cells were fixed with acrolein/formaldehyde and stained with the phospho-MAP kinase antibody that was then visualized with a secondary antibody conjugated to Texas red. The intensity of the immunofluorescent signal increased substantially after stimulation in both cases, and the activated MAP kinase was observed to concentrate in the nucleus, as previously reported (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993). The degree of nuclear concentration of the activated MAP kinase was more evident in the NIH3T3 than in the PtK1 cells.

The specificity of the phospho-antibody for the individual MAP kinase phosphoresidues was determined using a panel of cell lines expressing various phosphorylation site mutants of ERK2 tagged with the HA epitope: wild-type
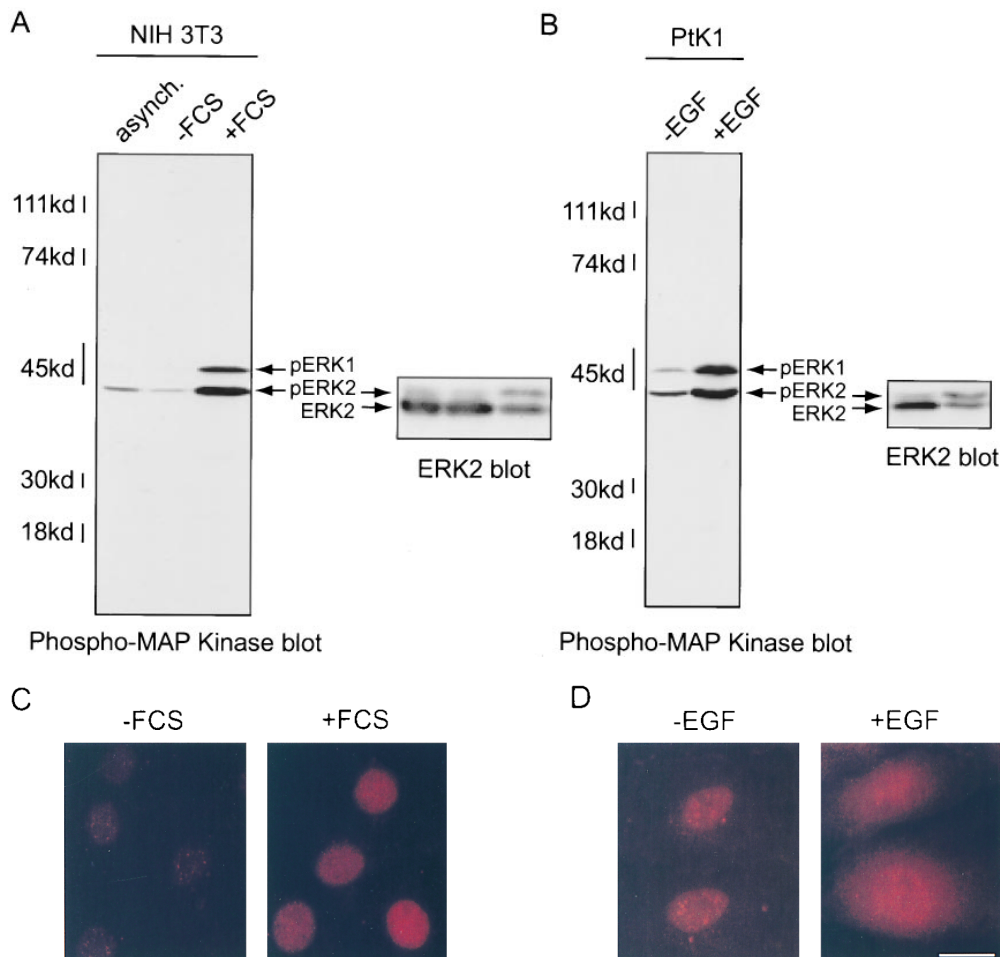

Figure 1. Specificity of phospho-MAP kinase antibody. (*A*) Phospho-MAP kinase immunoblot of whole cell lysate from NIH3T3 fibroblasts maintained in 10% FCS containing media (*asynch.*); serum deprived (0.2% FCS) for 25 h (− *FCS*) or serum deprived and then stimulated for 15 min with 20% FCS (+ *FCS*). *Right*, corresponding ERK2 monoclonal blot, showing the total amount of phosphorylated and unphosphorylated ERK2. (*B*) Phospho-MAP kinase immunoblot of whole cell lysate from PtK1 epithelial cells maintained in media with 10% FCS (− *EGF*) or EGF stimulated (0.2 µg/ml; 15 min) (+ *EGF*). Next to it is the corresponding monoclonal ERK2 antibody blot. (*C*) Indirect immunofluorescence of acrolein/formaldehyde fixed and phospho-MAP kinase antibody labeled NIH3T3 cells that were either serum deprived (0.2% FCS) for 25 h (− *FCS*) or serum stimulated (20% FCS) for 15 min (+ *FCS*). (*D*) The corresponding immunofluorescence of PtK1 cells either maintained in 10% FCS media (− *EGF*) or stimulated with EGF (0.2 µg/ml; 15 min) (+ *EGF*). Bar, 10 µm.
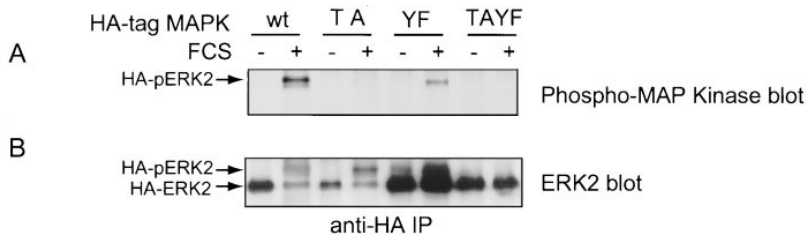
Figure 2. Specificity of phospho-MAP kinase antibody for the dually phosphorylated form of ERKs. (*A*) Phospho-MAP kinase immunoblot of hemagglutinin (*HA*) immunoprecipitated wild-type HA-ERK2 (*wt*) and HA-ERK2 phosphorylation site mutants (T183A [*TA*]; Y185F [*YF*]; T183A/Y185F [*TAYF*]) from stable expressing CCL39 fibroblasts. The cells were grown in 10% FCS containing media and then were either serum deprived (0.2% FCS) for 25 hours (− *FCS*) or serum deprived and then stimulated (20% FCS) for 5 min (+ *FCS*). (*B*) The corresponding ERK2 monoclonal immunoblot of the same membrane. Proteins were visualized by enhanced chemiluminescence.

(wt), T183A (TA), Y185F (YF), and the double mutant T183A/Y185F (TAYF) (Her et al., 1993). The cells were serum deprived and left untreated or serum stimulated and the tagged ERKs were immunoprecipitated with a HA-specific antibody. The immunoprecipitated proteins were resolved on SDS gels and their recognition by phospho-MAP kinase antibody was then assessed by Western blotting (Fig. 2 *A*). The antibody reacted strongly only with the wild-type enzyme from serum-stimulated cells. Weaker reactivity was also seen in the YF mutant, but only when the amount of MAP kinase loaded was in great excess compared with the other mutants (Fig. 2 *B*). As shown previously (Her et al., 1993), phosphorylation at either T183 or Y185 is sufficient to cause MAP kinase to display an electrophoretic mobility shift, and the double TAYF mutant did not shift at all. These data demonstrate that our phospho-MAP kinase antibody does not react detectably with unphosphorylated or tyrosine-phosphorylated MAP kinase, weakly with the threonine phosphorylated enzyme and strongly with the dually phosphorylated and activated MAP kinase. Reactivity with this antibody

thus is indicative of an activated enzyme, and can be used as a surrogate for enzyme assays.

Phospho-MAP Kinase Localizes at Kinetochores, Asters, and Midbody during Somatic Cell Mitosis

The localization of active MAP kinase during mitosis was determined by indirect immunofluorescence analysis of PtK1 cells. These cells were selected for study because of their large size, small chromosome number, and flat morphology during mitosis. Initially, we used cells treated with vinblastine to increase the mitotic yield. When mitotic cells were fixed with formaldehyde or acrolein/formaldehyde and then probed with anti–phospho-MAP kinase and Texas red-conjugated secondary antibody, the fluorescent signal was broadly distributed throughout the cell (data not shown). However, there were concentrations of increased intensity of staining, suggesting that activated MAP kinase might be concentrated (although not exclusively located) at specific subcellular structures.
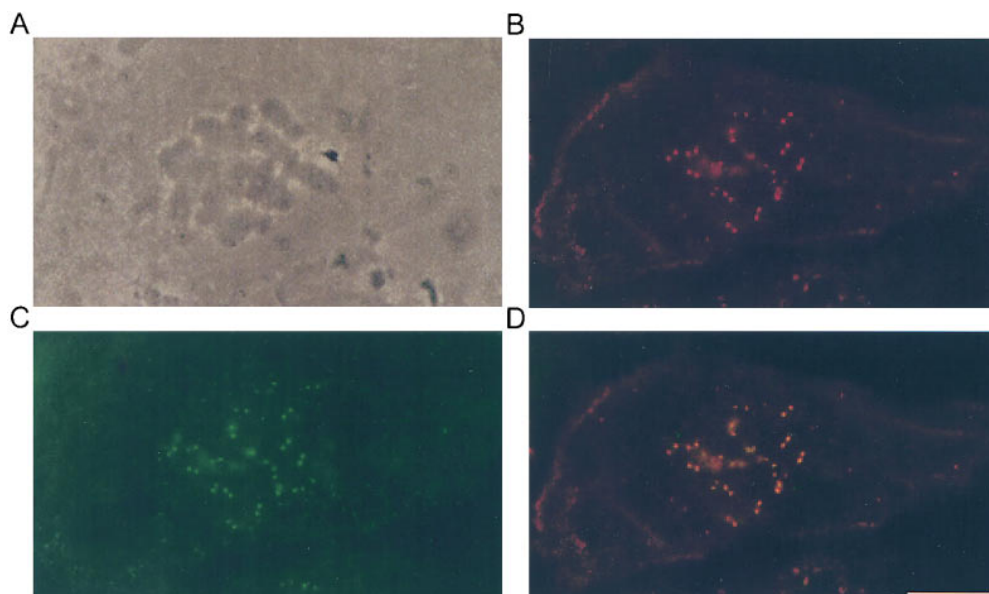

Figure 3. Phospho-MAP kinase localization at kinetochores of a vinblastine-treated cell. Indirect immunofluorescence was applied on this vinblastine treated, extracted, and fixed PtK1 cell. It was double labeled with phospho-MAP kinase antibody (*red*) and CREST autoimmune serum (*green*). (*A*) Phase contrast; (*B*) phospho-MAP kinase antibody; (*C*) CREST serum; (*D*) double labeling picture. In the latter, the kinetochores are seen in *yellow*, indicating colocalization of CREST and phospho-MAP kinase signal. The paired dot signal of the kinetochores is due to kinetochore labeling of sister chromatids. Note that no phospho-MAP kinase aster labeling is observed when the mitotic spindle is disrupted. Bar, 10 μm.

To reveal the subcellular locations where activated MAP kinase was concentrated, vinblastine-treated PtK1 cells arrested in mitosis were first extracted with CHAPS detergent, then formaldehyde fixed and labeled with phospho-MAP kinase antibody. The phospho-MAP kinase signal appeared as paired dots that resembled kinetochore labeling of sister chromatids (Fig. 3 *B*). To determine whether these paired dots were in fact kinetochores, the cells were also labeled with CREST autoimmune serum, which reacts with kinetochore proteins (Earnshaw and Rothfield, 1985). The CREST signal was visualized with FITC-conjugated secondary antibody, which displayed paired dots apparently identical to those seen with the phospho-MAP kinase antibody (Fig. 3 *C*). Double labeling with the two fluorophores yielded yellow paired dots, indicating colocalization of the phospho-MAP kinase and CREST signals (Fig. 3 *D*). Thus, activated MAP kinase is localized to kinetochores during prometaphase in vinblastine-treated cells.
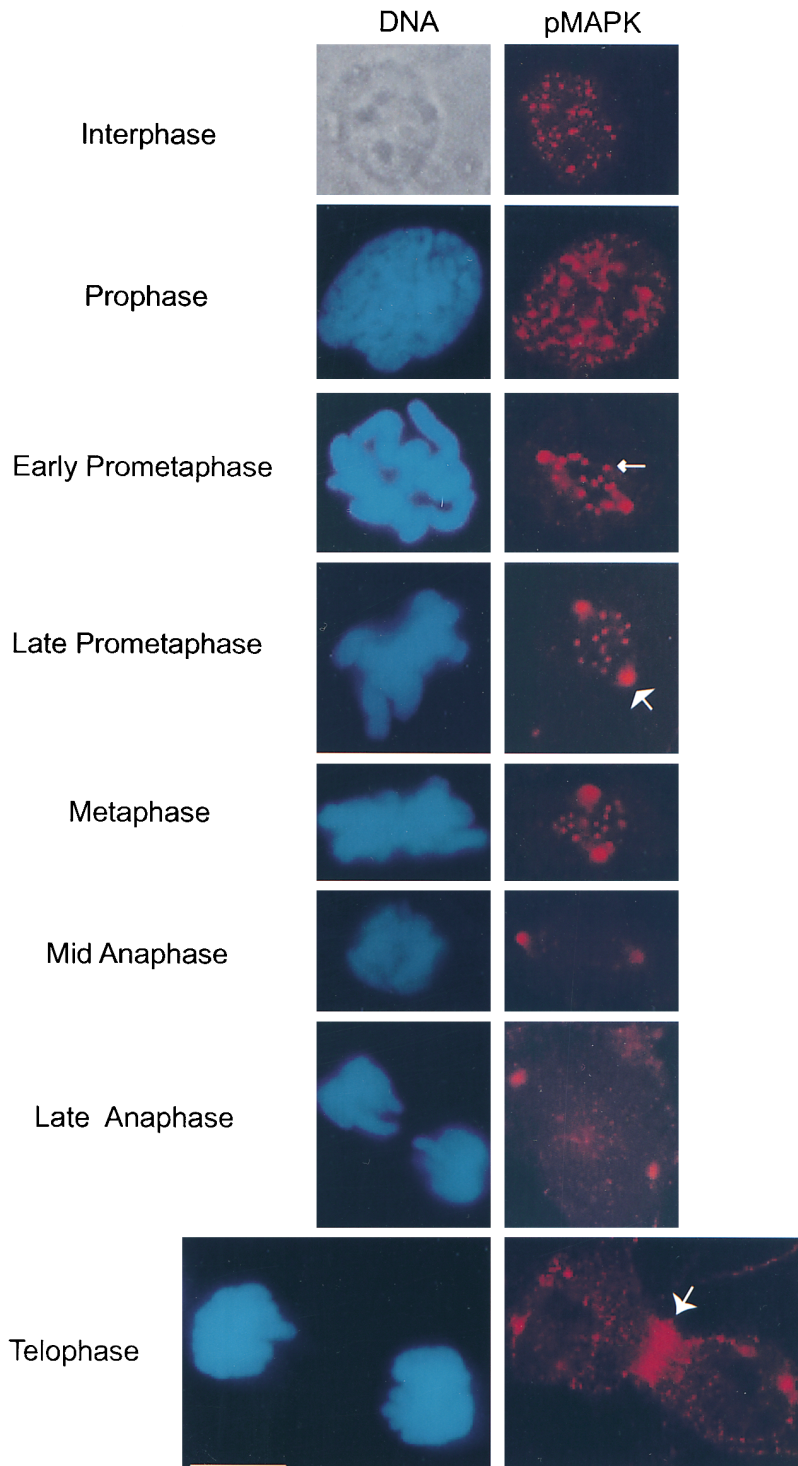
To determine whether the kinetochore localization of

Figure 4. Localization of phospho-MAP kinase during different stages of mitosis. Indirect immunofluorescence was performed on 1% Chaps extracted and 1% formaldehyde fixed PtK1 epithelial cells. The cells were labeled with the phospho-MAP kinase antibody (*red*) and counterstained with the DNA dye, DAPI (*blue*). In one case (interphase stage), the phase-contrast picture is shown instead of DAPI. *Arrow*, kinetochores in early prometaphase, asters in late prometaphase, and midbody in telophase. Bar, 10 μm.

MAP kinase was artificially induced by the microtubule-disrupting agent, we examined the location of phospho-MAP kinase in cells at various stages of mitosis in asynchronous cultures (Fig. 4). In interphase, phospho-MAP kinase had a punctate nuclear staining and was excluded from the nucleolus. In prophase, phospho-MAP kinase labeling was similar to the interphase stage, but the punctate character was less sharply defined. In prometaphase the pattern of phospho-MAP kinase labeling changed drastically: the punctate pattern disappeared and the labeling was present only as doublets corresponding to kinetochores of sister chromatids and in asters. (Note that aster labeling was not observed in the vinblastine-treated cell shown in Fig. 3 in which the microtubules were depolymerized). The kinetochore labeling appeared to be stronger in early than in late prometaphase, suggesting a decrease in phospho-MAP kinase signal as chromosomes congress toward the equatorial plate. At metaphase the phospho-MAP kinase staining at asters was maintained, whereas the kinetochore staining was slightly diminished. Aster staining persisted while kinetochore staining continued to drop as cells progressed into anaphase with kinetochore staining being virtually absent by midanaphase. During telophase and cytokinesis aster labeling was maintained and was accompanied by substantial staining of the contractile ring or midbody. Finally, after cytokinesis the reformed nuclei displayed the characteristic punctate interphase staining seen in exponential cultures (data not shown). Thus, it is clear that the appearance of activated MAP kinase at various subcellular sites during mitosis is regulated either by localization or by a phosphorylation and dephosphorylation cycle.

Whereas active MAP kinase signal at the kinetochores was absent in interphase and prophase, the CREST labeling was localized at kinetochores in both instances (data not shown). We cannot distinguish between the possibility that the active enzyme is not at the kinetochores during those cycle stages or that the phosphoepitope is somehow masked and therefore not recognized by the antibody.

Active MAP kinase was detected at kinetochores only when tyrosine and serine/threonine phosphatase inhibitors (vanadate and β glycerol-phosphate respectively) were included in the extraction solution. When the inhibitors were omitted, the kinetochore labeling was undetectable (data not shown). This suggests that the antibody reactivity depends on a phosphoepitope.

The kinetochore phospho-MAP kinase labeling was also detected with other fixation methods (acrolein/formaldehyde), as well as with antibody against total ERK2. In the former case, the background labeling was very high, which obscured the visualization of the kinetochore labeling. In the latter case, the kinetochore labeling was evident only if cells were previously treated with vinblastine, a procedure that maximized the phospho-MAP kinase signal.

Specificity of Phospho-MAP Kinase Labeling at Kinetochores

Although the phospho-specific antibody was highly specific on Western blots, it was important to verify its specificity in immunocytochemical procedures. To test this, we determined that the signal could be blocked with the anti-
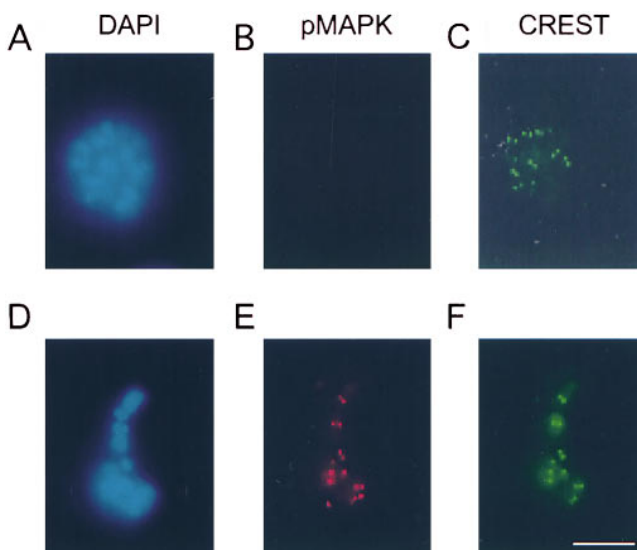

Figure 5. Inhibition of phospho-MAP kinase antibody by blocking with the phosphopeptide. Vinblastine-treated PtK1 cells were extracted and fixed, after which indirect immunofluorescence was performed. The cell in *A–C* was blocked with the phosphopeptide CHTGFLpTEpYVATR, whereas the cell shown in *D–F* was blocked with the corresponding nonphosphopeptide CHTG-FLTEYVATR. (*A* and *D*) DAPI staining; (*B* and *E*) labeling of phospho-MAP kinase antibody; (*C* and *F*) CREST autoimmune serum labeling. Both peptides were used at 10-fold excess mol/mol over the antibodies. Bar, 10 μm.

genic phosphopeptide (CHTGFLpTEpYVATR) but not the cognate unphosphorylated peptide (Fig. 5, *B* and *E*). Note that the phosphopeptide, while blocking reactivity of the cells with phospho-MAP kinase antibody, had no effect on reactivity with CREST antibody (Fig. 5, *C* and *F*). Comparable blocking of immunoreactivity was obtained when phosphorylated and activated recombinant MAP kinase was used as the blocking agent. Similarly, nonphosphorylated, inactive MAP kinase did not block immunoreactivity (data not shown). No immunoreactivity was detected with preimmune serum or with primary or secondary antibodies alone (data not shown). Additionally, microinjection of the phospho-MAP kinase antibody into mitotic cells localized the antibody on kinetochores and asters (data not shown). Thus, this antibody is as specific for phosphorylated and activated MAP kinase in immunocytochemical assays as it is in Western blots.

Phospho-MAP Kinase Localizes at Kinetochores of Isolated Chromosomes

To confirm the kinetochore localization of phospho-MAP kinase, we examined its localization on isolated chromosomes prepared from mitotic cells. Colcemid-arrested HeLa S3 cells were used for chromosome purification. Immunofluorescence was performed on formaldehyde-fixed chromosomes using phospho-MAP kinase antibody and CREST autoimmune serum. The phospho-MAP kinase localized at the centromeric region of the chromosomes, resembling the CREST signal (Fig. 6, *B* and *C*). Kinetochore staining was also seen with an anti-ERK2 polyclonal
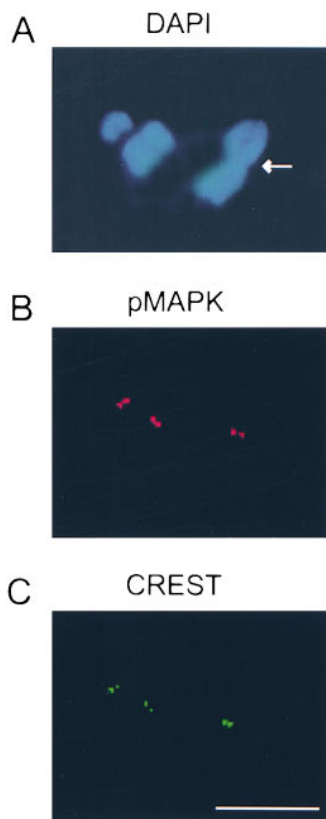
A DAPI

B pMAPK

C CREST

Figure 6. Localization of phospho-MAP kinase on kinetochores of isolated chromosomes. Chromosomes were obtained from colcemid-arrested HeLa S3 cells, which were pelleted onto coverslips and fixed with 1% formaldehyde. Immunofluorescence was immediately performed. (*A*) DAPI staining; (*B*) phospho-MAP kinase antibody labeling; (*C*) CREST autoimmune serum labeling. *Arrow*, centromeric region of a chromosome to which phospho-MAP kinase antibody and CREST serum bind, as seen in *B* and *C*. Bar, 10 μm.

rabbit serum that reacts with both phosphorylated and unphosphorylated MAP kinase (data not shown).

CENP-E Is an In Vitro MAP Kinase Substrate

To identify candidate substrates for kinetochore-associated MAP kinase, we looked for known mitotic proteins that show the same localization pattern as the phospho-MAP kinase, have MAP kinase consensus phosphorylation sites and are phosphorylated in mitosis. Only the kinetochore-associated motor protein CENP-E is known to display a localization pattern similar to phospho-MAP kinase. CENP-E is first detected at kinetochores at prometaphase (after nuclear envelope breakdown) and stays at the kinetochores until midanaphase, when its kinetochore association diminishes. It then is prominently detected at the spindle midzone in late anaphase, after which at telophase/cytokinesis it is present in the midbody (Yen et al., 1991, 1992; Cooke et al., 1997). CENP-E is also reported to be phosphorylated during mitosis (Liao et al., 1994) and was recently shown to be essential for chromosome alignment (Schaar et al., 1997; Wood et al., 1997).

Both human and frog CENP-E contain a COOH-terminal MAP kinase consensus site P-X-S/T-P (serine 2,570 in human and threonine 2,782 in frog), as well as several XSP sites that are consensus sites for the cdc2 kinase, but can be potential phosphorylation sites for MAP kinase as well. The COOH-terminal portion (amino acids 2,295–2,663; ~45 kD) of the human CENP-E is specifically phosphorylated in vivo during mitosis and in vitro by cdc2 kinase (Liao et al., 1994). This phosphorylation decreases the mi-

crotubule binding ability of the COOH-terminal domain of CENP-E in vitro (Liao et al., 1994). The corresponding COOH-terminal CENP-E mutant (CENP-E4A) that has alanines instead of serines in the XSP consensus (serines 2,567, 2,570, 2,601, and 2,616) is neither phosphorylated in vivo during mitosis nor in vitro by cdc2 kinase (Yen, T.J., unpublished results).

To determine whether MAP kinase can phosphorylate CENP-E, an in vitro phosphorylation assay was performed using recombinant activated MAP kinase and recombinant wild-type CENP-E or the serine to alanine mutant (CENP-E4A) of the COOH-terminal portion of human CENP-E protein (Fig. 7). The results indicate that the wild-type COOH-terminal CENP-E protein could be phosphorylated by MAP kinase whereas the CENP-E4A mutant was not. None of the reactions without active MAP kinase showed CENP-E phosphorylation, indicating that the phosphorylation of the wild-type CENP-E was specifically due to MAP kinase and not a contaminant in the CENP-E preparation. All four single-site serine to alanine CENP-E mutants could be phosphorylated in vitro with active MAP kinase and no site preference was observed when phosphopeptide mapping was performed on the wild-type COOH-terminal CENP-E phosphorylated in vitro by MAP kinase (data not shown). The same kinetics of phosphorylation and phosphopeptide maps were obtained by phosphorylating the COOH-terminal domain of CENP-E in vitro by cdc2 kinase (Liao et al., 1994), indicating that the two kinases phosphorylate the COOH terminus at the same sites with no preference. Overall, this result suggests that MAP kinase, as well as cdc2 kinase, can phosphorylate the COOH-terminus of CENP-E in vitro on sites known to regulate its microtubule binding ability.

MAP Kinase Associates with CENP-E In Vivo

Because CENP-E is an in vitro MAP kinase substrate and colocalizes with active MAP kinase at different stages of mitosis, we tested whether CENP-E and MAP kinase interact in vivo. CENP-E (315 kD) or MAP kinase (42 kD) were immunoprecipitated from asynchronous or mitotic HeLa cell lysates, separated on a 4 →12% SDS gradient gel, and then the coimmunoprecipitating partner was analyzed by Western blotting (Fig. 8). CENP-E immunoprecipitation showed more CENP-E in mitotic than in inter-
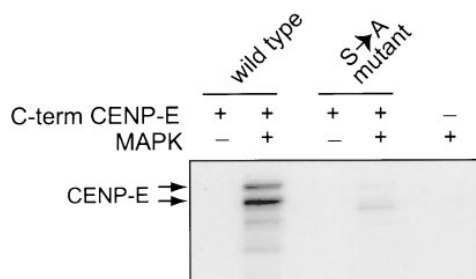
	wild type		S→A mutant		
C-term CENP-E	+	+	+	+	−
MAPK	−	+	−	+	+
CENP-E					

Figure 7. CENP-E is an in vitro MAP kinase substrate. Autoradiograph of a MAP kinase phosphorylation assay using a recombinant wild-type or the corresponding serine to alanine mutant of the COOH-terminal fragment of human CENP-E protein. Both bands are CENP-E, the lower being a degradation product.
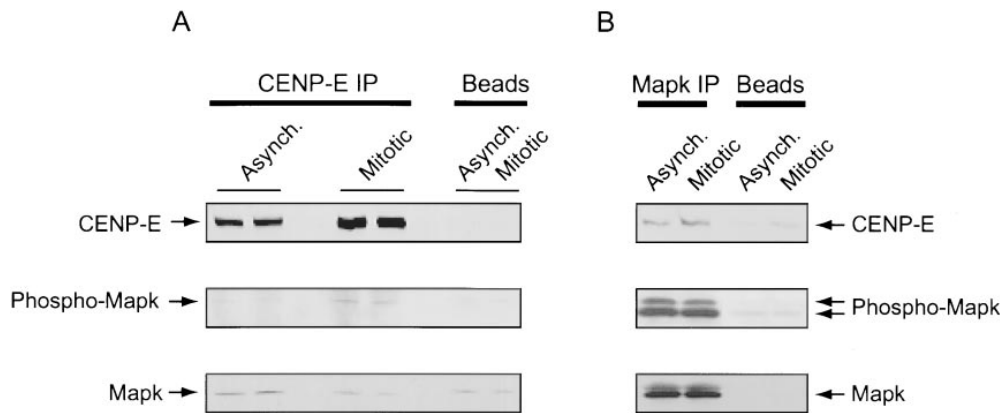
Figure 8. CENP-E associates with active MAP kinase during mitosis. Asynchronous or nocodazole-treated HeLa cells were lysed in a hypotonic buffer after which immunoprecipitation was performed using a CENP-E antibody, MAP kinase antibody, or beads alone. The immunoprecipitated material was subjected to SDS-PAGE (4–12% gel), followed by transfer to nitrocellulose and Western blotting with appropriate antibodies. (*A*) CENP-E immunoprecipitations. Here, single lanes are shown for the bead controls, whereas duplicate lanes are shown for the immunoprecipitations. (*B*) MAP kinase immunoprecipitations. In both *A* and *B*, CENP-E blot, phospho-MAP kinase blot, and ERK2 monoclonal antibody blot (for which the membrane was previously stripped) is shown. The proteins were visualized by enhanced chemiluminescence.

phase cells in agreement with previous findings (Yen et al., 1992). The phospho-MAP kinase antibody blot revealed that active MAP kinase coimmunoprecipitated with CENP-E and that the coimmunoprecipitation from mitotic lysates was greater than from interphase lysates (Fig. 8 *A*), even though more phospho-MAP kinase was present in the interphase lysates (data not shown). Reprobing the membrane with an anti-ERK2 monoclonal antibody showed unphosphorylated MAP kinase preferentially coimmunoprecipitated with CENP-E from lysates of asynchronous cells. Immunoprecipitation of total MAP kinase from cell lysates revealed coimmunoprecipitation of CENP-E (Fig. 8 *B*). In this case there was no difference between interphase and mitosis in the amount of coprecipitating CENP-E protein, presumably because the immunoprecipitating anti-MAP kinase antibody did not distinguish between phosphorylated and nonphosphorylated forms of the enzyme. These data indicate that CENP-E and MAP kinase interact in vivo and that this interaction with the phospho-MAP kinase form is regulated with the cell cycle.

Discussion

Active MAP Kinase Localizes at Kinetochores, Asters, and Midbody during Mitosis

In this manuscript we demonstrate that during mitosis a portion of the phosphorylated and activated MAP kinase is found at kinetochores, asters, and the midbody. The kinetochore localization was first detected and is the strongest at early prometaphase (after nuclear envelope breakdown). It was visible at a reduced level in later prometaphase stages and metaphase, after which it decreased substantially as anaphase initiated and became undetectable in midanaphase. This kinetochore localization pattern is consistent with a possible role for MAP kinase in microtubule capture and chromosome congression in early and midmitotic stages. The temporal pattern in which the kinetochore phospho-MAP kinase signal diminishes as anaphase is initiated is reminiscent of the pattern seen or inferred

with other phosphoepitopes (e.g., 3F3/2 and MPM-2) (Gorbsky and Ricketts, 1993; Taagepera et al., 1995). Thus, the dephosphorylation of several proteins is associated with anaphase onset and is believed to be required for mitotic progression.

Asters showed localization of active MAP kinase in all mitotic stages except prophase. Previous work on mouse oocytes showed MAP kinase at the microtubule organizing centers (Verhlac et al., 1993). Several centrosomal proteins that have MAP kinase phosphorylation consensus sites, such as pericentrin (Laird and Shalloway, 1997) and nuclear mitotic apparatus protein (NUMA) (Sparks et al., 1995), are also known to be phosphorylated specifically at mitosis. Interestingly, pericentrin seems to be phosphorylated by a kinase that is different from cdc2 kinase (Laird and Shalloway, 1997). Thus, active MAP kinase might have an additional role in the assembly and stability of the centrosomes during mitosis.

At midanaphase, the phospho-MAP kinase signal disappears from kinetochores. It appears at the fibrous area at the original midzone region in late anaphase and then localizes at the midbody during telophase and cytokinesis. Like MAP kinase, inner centromere protein (INCENP) (Cooke et al., 1987), CENP-E (Yen et al., 1991), and CENP-F (Rattner et al., 1993; Zhu et al., 1995) translocate from kinetochores to the midbody at the end of mitosis. INCENP translocation has been shown to be essential for proper cytokinesis (Eckley et al., 1997). Therefore, the movement of active MAP kinase to the midbody raises the possibility that the enzyme could also play a role in anaphase B and cytokinesis.

Our localization findings suggest that active MAP kinase plays a role in normal mitotic progression. This seems to contradict some previous reports suggesting that MAP kinase activity can disrupt progression through M phase. For example, sustained MAP kinase activation is necessary for the spindle checkpoint in nocodazole-treated *Xenopus* egg extracts (Minshull et al., 1994; Takenaka et al., 1997) and can cause either a G_2 or M phase arrest in the first mitotic cell cycle in *Xenopus* (Abrieu et al., 1997;

Walter et al., 1997; Bitangcol et al., 1998). How could one enzyme function both as a component of normal M phase progression and as an effector of mitotic arrest? It is possible that passage from one M phase to another requires cycles of phosphorylation and dephosphorylation of proteins involved in chromosome movement and cell cycle progression. On the other hand, sustained phosphorylation of these proteins might result in M phase arrest. Whether specific localizations of activated MAP kinase reported here play a role in M phase arrest remains to be determined.

Although MAP kinase plays a role in the M phase arrest in *Xenopus* egg extracts and early embryos, its ability to induce M phase arrest in somatic cells is less certain. When injected into somatic cells, Mos (an in vitro MEK activator) localized to kinetochores and induced a metaphase-like arrest (Wang et al., 1994). Although it is clear from this study that the effect was due to enzymatically activated Mos, it was not shown that its effect on M phase progression was due to its kinetochore localization and/or activation of MAP kinase. Similarly, injection of MAP kinase phosphatase CL100 into nocodazole-treated XTC cells reversed the mitotic arrest (Wang et al., 1997). However, no determination was made whether MAP kinase was inactivated in these experiments. Moreover, a recent report implicating the MAP kinase homologue p38 in M phase arrest in mammalian cells (Takenaka et al., 1998) and the fact that CL100 seems to have higher activity for dephosphorylation of p38 than ERKs (Chu et al., 1996) raises a question about which of these kinases plays the predominant role in nocodazole-induced mitotic arrest. It is possible that in somatic cells the MAP kinases ERK1 and ERK2 are important for normal mitotic progression, whereas p38 kinase functions in the spindle checkpoint.

MAP Kinase Phosphorylates and Associates with the Motor Protein CENP-E

To gain insight into possible functions of MAP kinase in mitosis, we looked for proteins important in mitosis that might be candidate MAP kinase substrates, using the following criteria: (*a*) localization during mitosis similar to phospho-MAP kinase; (*b*) presence of MAP kinase consensus phosphorylation sites; and (*c*) functionally significant mitotic phosphorylation. Using these criteria we identified the kinetochore motor protein CENP-E as a candidate MAP kinase substrate. The reported localization of CENP-E during mitosis coincides with that of phospho-MAP kinase: it is predominately at the kinetochore from early prometaphase until midanaphase and at the midbody during telophase/cytokinesis (Yen et al., 1991, 1992; Cooke et al., 1997). The *Xenopus* homologue of CENP-E is additionally found at the asters, as is MAP kinase (Wood et al., 1997). Both human and frog CENP-E contain MAP kinase consensus phosphorylation sites at their COOH terminus. Finally, the human CENP-E is reported to be phosphorylated in vivo during mitosis (Liao et al., 1994).

CENP-E is a kinesin-related motor protein whose kinetochore localization during prometaphase is essential for bipolar attachment of chromosomes to microtubules and for proper chromosome congression toward the meta-

phase plate (Shaar et al., 1997; Wood et al., 1997). The human CENP-E has two microtubule-binding domains: one at the NH_2 terminus whose binding is ATP dependent and the second one at the COOH terminus whose binding is ATP independent. The latter domain has four potential MAP kinase and cdc2 phosphorylation sites, and was shown to be phosphorylated in vivo during mitosis and in vitro by cdc2 kinase (Liao et al., 1994).

We find that CENP-E can be a MAP kinase substrate in vitro. Phosphopeptide maps of the COOH-terminal CENP-E phosphorylated in vitro by MAP kinase (data not shown) revealed the same sites as the COOH terminus of CENP-E phosphorylated in vivo during mitosis and in vitro by cdc2 (Liao et al., 1994). Since the consensus phosphorylation sites for MAP kinase and cdc2 kinase are similar and both enzymes can phosphorylate CENP-E in vitro with similar kinetics and site preference, it is not possible to be certain which of the kinases is responsible for in vivo CENP-E phosphorylation or whether both are physiologically relevant CENP-E kinases. In vitro, CENP-E seems to be at least as good if not better substrate for MAP kinase than cdc2 (data not shown). We suspect that CENP-E is a convergence point for regulatory phosphorylation by several differentially regulated kinases.

We also find that CENP-E and MAP kinase associate in vivo. Immunoprecipitation of CENP-E coprecipitates MAP kinase and vice versa. The phospho-MAP kinase is preferentially coimmunoprecipitated from mitotic extracts, and unphosphorylated MAP kinase is preferentially coimmunoprecipitated from interphase extracts. Thus, it is clear that the association of active MAP kinase and CENP-E is regulated with the cell cycle. We do not know whether MAP kinase binds directly to CENP-E or is part of a larger complex.

The requirement for and functions of CENP-E phosphorylation are not well understood. The COOH-terminal domain of CENP-E, when phosphorylated in vitro, showed decreased microtubule binding ability (Liao et al., 1994). This suggests that in vivo, CENP-E binding to microtubules via its COOH-terminal domain might depend on its phosphorylation status. The COOH-terminal domain of CENP-E expressed in mitotic cells localized to the spindle during anaphase but not metaphase, and this is hypothesized to reflect changes in the phosphorylation state of CENP-E (Liao et al., 1994). At the kinetochore, CENP-E could potentially bind microtubules via its NH_2-terminal domain throughout mitosis (the binding is ATP dependent and apparently phosphorylation independent), whereas the binding to microtubules via its COOH-terminal domain would depend on its phosphorylation status. This implies that in early mitotic stages, when the COOH terminus of CENP-E is phosphorylated, one molecule of CENP-E will bind via its NH_2-terminal domain to only one microtubule. This could be important in the initial phases of microtubule capture by kinetochores and in congression. Cycles of phosphorylation and dephosphorylation of the CENP-E COOH-terminus as congression proceeds might additionally allow the speed and directionality of chromosome movement to be titrated, so that chromosomes could align at the metaphase plate with minimal entanglements. In anaphase B when the COOH terminus of CENP-E is unphosphorylated, CENP-E will bind microtu-

bules via both its NH$_2$- and COOH-terminal domains. At the kinetochore, microtubule binding by the COOH terminus of CENP-E may be important for stabilizing kinetochore–microtubule interactions during chromatid separation. In addition, CENP-E is also localized to the spindle midzone where it may use its two microtubule binding domains to cross-link antiparallel microtubules, stabilizing the spindle and facilitating pole and chromosome separation.

During G$_o$ transitions, MAP kinase phosphorylates numerous substrates, including receptors, signaling adapters, transcription factors, and other kinases (L'Allemain, 1994). It is believed that by phosphorylating multiple substrates this effector kinase can orchestrate a coordinated but functionally diverse response to extracellular agonists. Similarly, we expect that during M phase MAP kinase will have multiple functionally significant substrates. Since only a portion of the activated MAP kinase is found at kinetochores, asters, and midbody (data not shown), some of these additional substrates might not be associated with these structures. It should be noted that MAP kinase was originally identified as phosphorylating microtubule-associated protein-2 (Ray and Sturgill, 1987), and that this phosphorylation decreases its microtubule stabilizing activity (Hoshi et al., 1992). Likewise, active MAP kinase added to interphase frog egg lysates induces a decrease in microtubule half-life and forms an M phase microtubule pattern (Gotoh et al., 1991). Studies are underway to find additional mitotic MAP kinase substrates and to determine what changes in mitosis occur when MAP kinase is ectopically activated or inactivated.

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