Activation of the MKK/ERK Pathway during Somatic Cell Mitosis: Direct Interactions of Active ERK with Kinetochores and Regulation of the Mitotic 3F3/2 Phosphoantigen

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Abstract. The mitogen-activated protein (MAP) kinase pathway, which includes extracellular signal–regulated protein kinases 1 and 2 (ERK1, ERK2) and MAP kinase kinases 1 and 2 (MKK1, MKK2), is well-known to be required for cell cycle progression from G1 to S phase, but its role in somatic cell mitosis has not been clearly established. We have examined the regulation of ERK and MKK in mammalian cells during mitosis using antibodies selective for active phosphorylated forms of these enzymes. In NIH 3T3 cells, both ERK and MKK are activated within the nucleus during early prophase; they localize to spindle poles between prophase and anaphase, and to the midbody during cytokinesis. During metaphase, active ERK is localized in the chromosome periphery, in contrast to active MKK, which shows clear chromosome exclusion. Prophase activation and spindle pole localization of active ERK and MKK are also observed in PtK_1 cells. Discrete localization of active ERK at kinetochores is apparent by early prophase and during prometaphase with decreased staining on chromosomes aligned at the metaphase plate. The kinetochores of chromosomes displaced from the metaphase plate, or in microtubule-disrupted cells, still react strongly with the active ERK antibody. This pattern resembles that reported for the 3F3/2 monoclonal antibody, which recognizes a phosphoepitope that disappears with kinetochore attachment to the spindles, and has been implicated in the mitotic checkpoint for anaphase onset (Gorbsky and Ricketts, 1993. *J. Cell Biol.* 122:1311–1321). The 3F3/2 reactivity of kinetochores on isolated chromosomes decreases after dephosphorylation with protein phosphatase, and then increases after subsequent phosphorylation by purified active ERK or active MKK. These results suggest that the MAP kinase pathway has multiple functions during mitosis, helping to promote mitotic entry as well as targeting proteins that mediate mitotic progression in response to kinetochore attachment.

Key words: MAP kinase • mitosis • kinetochore • cell cycle • phosphorylation

Growth and differentiation factors regulate the mitogen-activated protein (MAP)[1] extracellular signal–regulated protein kinases 1 and 2 (ERK1 and ERK2) through pathways using receptor tyrosine kinases, cytokine receptors, and heterotrimeric G protein–coupled receptors (Lewis et al., 1998; Robinson and Cobb, 1997). Activation occurs by coupling receptors to Ras, Raf-1, and MAP kinase kinase-1 or -2 (MKK 1 or 2), the latter of which activates ERK directly through phosphorylation. MKK is also phosphorylated and activated by *c*-mos, a germ cell–specific protein kinase that activates the MKK/ERK pathway during meiotic cell division. ERK functions as an essential regulator of cell growth and differentiation in eukaryotic cells, so the identification of cellular substrates for this enzyme is an important goal in elucidating mechanisms for biological control.

The requirement for ERK in S phase entry is demonstrated by a G1 arrest in cells where ERK activation is blocked by expression of antisense constructs, dominant negative mutants, dual specificity phosphatases, and treat-

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1. *Abbreviations used in this paper*: APC, anaphase promoting complex; DAPI, 4′,6-diamidino-2-phenylindole; ERK, extracellular signal–regulated kinase; MAP, mitogen-activated protein; MKK, MAP kinase kinase; MEKK, MAP/ERK kinase kinase; PP2A, protein phosphatase type 2A; MPF, maturation promoting factor; NBF, neutral buffered formalin; NTA, Ni^{+2}-nitrilotriacetic acid.

ment with the inhibitor of MKK1, PD98059 (Dudley et al., 1995; Sun et al., 1993; Páges et al., 1993). ERK-regulated events required for progression into S phase include expression of immediate early genes such as Fos and Egr-1, and transcriptional upregulation of cyclin D1 (Lavoie et al., 1996; Kortenjann et al., 1994; Beno et al., 1995). However, the function for ERK in mitosis is less well-defined. The activations of *c*-mos, MKK, and ERK play important roles in inducing meiosis in oocytes (reviewed by Sagata, 1997), but ERK activity after fertilization declines and remains low during mitotic cell division in early embryos (Ferrell et al., 1991). Activation of ERK by *c*-mos or injection of active ERK from unfertilized eggs in metaphase II into two cell embryos induces cell cycle arrest, revealing a cytostatic factor activity of this enzyme (Kosako et al., 1994; Haccard et al., 1993). Likewise, adding active ERK to *Xenopus* cell free extracts leads to arrest in G2 and suppression of cyclin B/cdc2 activation (Abrieu et al., 1997; Walter et al., 1997). Taken together, these data suggest that ERK functions positively during meiotic cell division, but in fact negatively regulates mitotic progression in early embryos.

Consistent with results from early *Xenopus* embryos, previous reports in somatic mammalian cells have shown no activation of ERK during mitosis, as measured by SDS-PAGE gel mobility retardation or in-gel phosphorylation assays (Tamemoto et al., 1992; Edelmann et al., 1996). Ras also appears to remain inactive during mitosis (Taylor and Shalloway, 1996). Nevertheless, mitotic enhancement of Raf-1 activity in cells synchronized by mitotic shake-off or arrested with nocodazole has been reported (Laird et al., 1995; Pathan et al., 1996), and inhibition of c-Src by antibody microinjection blocks mitotic entry (Roche et al., 1995). These data indicate the existence of mitotic mechanisms for activating ERK through known upstream pathway components in somatic cells.

In this study, we examined the cellular localization of active ERK and MKK during mitosis using antibodies that specifically recognize active phosphorylated forms of these enzymes. We report the novel finding that ERK and MKK are activated early in prophase before nuclear envelope breakdown, then becoming localized at spindle poles later in prophase. Localization of ERK and MKK is not entirely overlapping, in that active MKK is excluded from condensed chromosomes, whereas active ERK associates with kinetochores and within the chromosomal periphery of condensed chromosomes. This result suggests that ERK phosphorylation by MKK may be involved in chromosomal targeting. A functional role for ERK as a sensor or effector for mitotic progression is suggested by correlations between the appearance and disappearance of active ERK at kinetochores, with those of the antigen(s) recognized by the 3F3/2 monoclonal antibody. Previous studies have shown that this antibody recognizes kinetochore phosphoantigens that respond to spindle fiber attachment (Gorbsky and Ricketts, 1993; Nicklas et al., 1995), and that microinjection of 3F3/2 antibodies delays anaphase entry, suggesting that the phosphoantigen is involved in regulating metaphase-to-anaphase transition (Campbell and Gorbsky, 1995). Our studies with isolated chromosomes indicate that the 3F3/2 epitope is directly or indirectly phosphorylated in response to ERK, suggesting novel roles for ERK in somatic cell mitosis.

Materials and Methods

Antibodies, Enzyme Purification, and Immunoblotting

Affinity-purified rabbit polyclonal antibody to diphosphorylated ERK2 (anti-ACTIVE MAPK) was purchased from Promega Corp. (Madison, WI), and mouse monoclonal antibody to diphosphorylated ERK2 was a generous gift of Dr. Rony Seger (Yung et al., 1997). In experiments performed to determine the specificity of the anti-ACTIVE MAPK antibody, wild-type or mutant $(His)_6$-rat ERK2 (Robbins et al., 1993) were expressed in bacteria, purified by Ni^{+2}-nitrilotriacetic acid (NTA) metal affinity chromatography (QIAGEN Inc., Valencia, CA), and phosphorylated for 10 min at 30°C with constitutively active mutant MKK1 (G1C: ΔN4/S218E/S222D; Mansour et al., 1996), which was expressed in bacteria and purified as described (Mansour et al., 1994). Reactions contained 1 μg ERK2, 1 μg MKK1, 0.1 mM ATP, 10 mM $MgCl_2$, 20 mM Hepes, pH 7.4, and 1 mM dithiothreitol in 25 μl. Alternatively, whole cell lysates were prepared from NIH 3T3 cells starved in DMEM, 0% FBS overnight, and then treated for 5 min with 10% serum and 0.1 μM PMA. Proteins were separated by SDS-PAGE, transferred to Immobilon *P* (Millipore Corp., Bedford, MA), reacted with primary antibody (1:1,000 dilution, 1 h) followed by 0.8 μg/ml goat anti–rabbit or mouse IgG (The Jackson Laboratories, Bar Harbor, ME) coupled to horseradish peroxidase, and then visualized by enhanced chemiluminiscence (Amersham Life Science, Inc., Arlington Heights, IL). Immunoblots were also probed using a rabbit polyclonal antibody recognizing the COOH terminus of ERK2 (C-14; Santa Cruz Biotechnology, Santa Cruz, CA).

Affinity-purified rabbit polyclonal antibody to phosphorylated MKK1/2 (anti-phosphoMEK1/2) was purchased from New England Biolabs Inc. (Beverly, MA). To test its specificity, wild-type $(His)_6$-human MKK1 was expressed in bacteria, purified by Ni^{+2}-NTA affinity chromatography (Mansour et al., 1996), proteolyzed with enterokinase (Invitrogen Corp., Carlsbad, CA), and phosphorylated with a constitutively active mutant of MEK kinase ($[His]_6$-MEKKΔC; Khokhlatchev et al., 1996) for 3 h with 4 mM ATP, 15 mM $MgCl_2$. This test resulted in monophosphorylated and diphosphorylated forms of MKK1 that were subsequently resolved by FPLC using a Mono Q HR5/5 column equilibrated in 20 mM Tris, pH 8, 10% (vol/vol) glycerol, 1 mM dithiothreitol, and were eluted with a linear sodium chloride gradient in the same buffer. Mass spectrometric peptide mapping and sequencing were performed using a PE Sciex API-III electrospray ionization mass spectrometer (Resing and Ahn, 1998). This analysis revealed that the monophosphorylated form of MKK1 was modified entirely at Ser218, indicating that phosphorylation by MEKK1 was ordered under these conditions. The diphosphorylated form of MKK1 was modified entirely at Ser218 and Ser222. Immunoblotting using anti-phospho-MEK1/2 antibody (1:1,000 dilution) was performed as above. Immunoblots were also probed with a rabbit polyclonal antibody recognizing the NH_2-terminus of MKK1 (C-18; Santa Cruz Biotechnology, Santa Cruz, CA).

For experiments with intact chromosomes, wild-type $(His)_6$-ERK2 or catalytically inactive $(His)_6$-ERK2(K52R) proteins were expressed in bacteria, purified by Ni^{+2}-NTA chromatography, and phosphorylated with constitutively active mutant MKK1 (G7B: ΔN4/S218D/S222D; Mansour et al., 1996), which was expressed in bacteria and proteolyzed with enterokinase to remove the $(His)_6$ tag. Reactions contained 170 μg ERK2, 15 μg MKK1-G7B, 4 mM ATP, 15 mM $MgCl_2$, 20 mM Hepes, pH 7.4, and 0.2% (vol/vol) β-mercaptoethanol in a final volume of 1 ml, and were incubated for 3 h at 30°C. ERK2 was purified away from MKK and ATP by adsorption to Ni^{+2}-NTA resin incubated for 20 min at room temperature, followed by three washes with 1 ml 10 mM Hepes, pH 7.4, 0.2% β-mercaptoethanol, and elution with 10 mM Tris pH 8, 0.3 M imidazole, 0.2% β-mercaptoethanol. Aliquots of the activated ERK2 were snap-frozen in liquid nitrogen and stored at −80°C. The reaction resulted in >90% diphosphorylation of ERK2 at Thr183 and Tyr185 as assessed by mass spectrometric peptide mapping and sequencing. The specific activity of the wild-type phosphorylated ERK2 was 1.5 μmol/min/mg, measured using 0.3 mg/ml myelin basic protein (Sigma Chemical Co., St. Louis, MO) and 0.1 mM ATP as substrates (Mansour et al., 1994).

Cell Culture and Immunofluorescence Analysis of Fixed Cells

Mouse NIH 3T3, CHO, or rat kangaroo PtK_1 cells were grown in DME-supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and

10% FBS (GIBCO-BRL, Gaithersburg, MD). In some experiments, cells were treated with nocodazole (5 μg/ml), for 30 min before examination.

Cells were grown to ~75% confluence on round glass cover slips in 6- or 10-cm plates. Cover slips were rapidly rinsed in cold (4°C) PBS, and were then immediately fixed under one of the following conditions: (*a*) glutaraldehyde fixation: samples were fixed with 0.1% (vol/vol) glutaraldehyde, 2% formaldehyde in PBS for 5 min, and were then permeabilized with PBS, 0.1% Triton X-100 for 5 min; (*b*) neutral buffered formalin (NBF)/methanol fixation: samples were fixed with 10% (vol/vol) NBF for 5 min, and were then permeabilized with cold (−20°C) 100% methanol for 5 min; (*c*) paraformaldehyde fixation: samples were fixed with 4% (vol/vol) paraformaldehyde for 5 min, and were then permeabilized with PBS, 0.1% Triton X-100 for 5 min. After fixation in all cases, cells were incubated in Tris-buffered saline (TBS, 50 mM Tris, pH 7.6, 0.15 M NaCl), 0.1% Tween 20, 3% BSA for 1 h. Coverslips were then incubated with primary antibody for 1 h, washed 4× with TBS, 0.1% Tween 20, incubated for 1 h with fluorescein isothiocyanate- or Texas Red–conjugated anti-rabbit or anti-mouse secondary antibody (0.8 μg/ml, The Jackson Laboratories), washed 4× with TBS, 0.1% Tween 20, and finally counterstained with 4′,6-diamidino-2-phenylindole (DAPI, 0.4 μg/ml in PBS). Fluorescent images were viewed and photographed using a Zeiss Axioplan fluorescence microscope with a Photometrics Sensys digital CCD camera system. Images were manipulated using IP-LAB Spectrum software. Controls for nonspecific staining were performed by preincubating primary antibody with 0.1 mg/ml diphosphorylated peptides CDPDHTGFLT(P)EY(P)VATRWYRA (used with antibody to phosphorylated ERK) or CVSGQLIDS(P)MANS(P)FVGTRSY (used with antibody to phosphorylated MKK), synthesized by Macromolecular Resources (Ft. Collins, CO).

Other primary antibodies used in immunocytochemical studies include: the mouse monoclonal antibody 3F3/2, which detects phosphorylated kinetochore epitopes (1:1,000 dilution; Cyert et al., 1988); a rabbit polyclonal antibody recognizing the kinetochore microtubule motor, CENP-E (1:200 dilution; Yen et al., 1991); a mouse monoclonal antibody recognizing lamin B used to monitor nuclear membrane integrity (1:200 dilution; Ab-1; Calbiochem-Novabiochem Corp., La Jolla, CA), and a mouse monoclonal antibody recognizing β-tubulin (1:10 dilution) used to monitor spindle pole location (Neighbors et al., 1988).

Purification, Immunostaining, and Analysis of Isolated Chromosomes

Mitotic CHO chromosomes were isolated as described by Hyman and Michison (1993, except that polyamines were omitted with no difference in results), and stored at −80°C in Pipes buffer (80 mM Pipes, pH 7, 1 mM EGTA, 4 mM $MgCl_2$, 5 mM dithiothreitol). Chromosomes were washed by fivefold dilution into Pipes buffer containing 1 mM benzamidine, 1 μg/ml aprotinin, and 0.5 μM phenylmethylsulfonyl fluoride, introduced into perfusion chambers constructed by placing glass coverslips onto two strips of cellophane tape (chamber volume ~10 μl), and allowed to settle by incubation on ice for 10 min (Hunt and McIntosh, 1998).

For immunochemistry, chromosomes were blocked by introducing 20 μl of 0.2 mg/ml BSA in Pipes buffer into the chamber and incubating for 2 min at room temperature. This was followed by 10 μl anti-ACTIVE ERK rabbit polyclonal antibody (1:100 dilution in Pipes buffer) or 3F3/2 monoclonal antibody (1:1,000 dilution in Pipes buffer) containing 0.4 μg/ml DAPI (2 min, room temperature). After two washes with 30 μl Pipes buffer, 20 μl fluorescein isothiocyanate- or rhodamine-conjugated anti-rabbit or anti-mouse secondary antibody (0.8 μg/ml) were introduced and incubated for 2 min, followed by a final wash with 30 μl Pipes buffer. Chambers were sealed with immersion oil, and observed directly without fixation.

For experiments examining effects of ERK, MKK, or protein phosphatases on immunostaining, 20 μl (2 U) of protein phosphatase type 2A catalytic subunit (PP2A; Promega Corp.) in Pipes buffer was introduced into perfusion chambers and incubated for 15 min at room temperature. This was followed by 20 μl Pipes buffer containing 2 μM microcystin (Alexis, Woburn, MA), and then 20 μl (2 μg) diphosphorylated ERK2-WT, ERK2-K52R, or constitutively active mutant MKK1-G7B in 25 mM Hepes, pH 7.4, 15 mM $MgCl_2$, 2 mM ATP, 2 μM microcystin, 1 mM dithiothreitol. Reactions were incubated for 10 min, and samples were blocked and treated with primary and secondary antibodies as described above. Control reactions were performed by adding microcystin to PP2A before its addition to sample, or by omitting ERK or MKK from the second reaction containing MgATP.

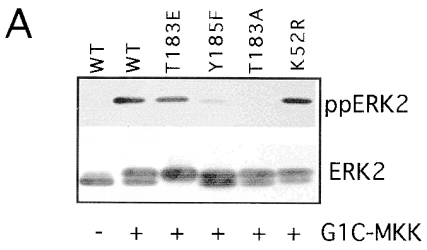
Samples were photographed by immunofluorescence microscopy as above, using a 100× objective, recording exposure times for each image. Chromosomes were visualized under each condition, and the fluorescence intensities of kinetochores were quantified by integrating absolute intensities within four pixel squares superimposed over the kinetochore pair. Pixel intensities were corrected by subtracting an instrument baseline intensity of 283 intensity units, determined by photographing a coverslip coated with uranium at varying exposures, and extrapolating to zero exposure time. Averages and standard errors of intensities are reported for 6–18 kinetochores as indicated.

Results

Wild-type ERK2 in different states of phosphorylation and four of its mutant alleles were examined by immunoblotting in order to establish the specificity of the polyclonal anti-ACTIVE MAPK antibody. Reactivity was observed with either the wild type or with a catalytically inactive mutant (K52R) after their phosphorylation with active MKK1 (Fig. 1 *A*, lanes *2* and *6*), but staining was completely absent with unphosphorylated ERK2 (Fig. 1 *A*, lane *1*). Reactivity was preferential for the diphosphorylated enzyme, since monophosphorylated ERK2-Y185F or -T183A mutants were poorly recognized by the antibody (Fig. 1 *A*, lanes *4* and *5*). Interestingly, antibody reactivity with monophosphorylated mutant ERK2-T183E was nearly as efficient as with diphosphorylated ERK2-WT, indicating the importance of a negative charge in epitope recognition (Fig. 1 *A*, lane *3*). Immunoblots of whole cell extracts using the anti-ACTIVE MAPK antibody showed elevated reactivity with ERKs 1 and 2 after stimulation of NIH 3T3, CHO, or PtK_1 cells with 10% serum/phorbol ester, consistent with the expected protein kinase activation (Fig. 1 *B*, *top*). Importantly, no other proteins within the extracts were strongly reactive, indicating specific recognition of ERKs among total cellular proteins. Comparable loading of samples was shown by immunoreactivity of the same cell extracts using a COOH-terminal ERK2 antibody (Fig. 1 *B*, *bottom*).

Immunocytochemical staining of the anti-ACTIVE MAPK antibody was examined in NIH 3T3 cells in various stages of mitosis. Cells in interphase showed little or no reactivity, but marked increases in active ERK staining were observed in the nuclei of prophase cells identified by the morphology of their chromatin (Fig. 2, *A* and *G*). Costaining of lamin B showed that the increased ERK reactivity during prophase occurred before nuclear envelope breakdown (Fig. 3). Cells in metaphase showed a distinct localization of active ERK to regions surrounding the condensed chromosomes (Fig. 2, *B* and *H*). Close comparison with DAPI staining indicated that active ERK staining mainly occurred in regions surrounding chromatin, while condensed chromosome arms were not as strongly reactive. Staining of active ERK was also found at both spindle poles (Fig. 2, *H* and *I*) whose location was confirmed by costaining with β-tubulin antibodies (data not shown). In anaphase, active ERK immunoreactivity was more diffusely localized and largely excluded from chromosomes (Fig. 2, *C* and *I*). In telophase/cytokinesis, active ERK appeared localized within cytoplasmic regions of both daughter cells as well as the midbody (Fig. 2, *D* and *J*).

The specificity of immunostaining was also tested by preincubating the primary antibody with a diphosphorylated peptide corresponding to the phosphoepitope on



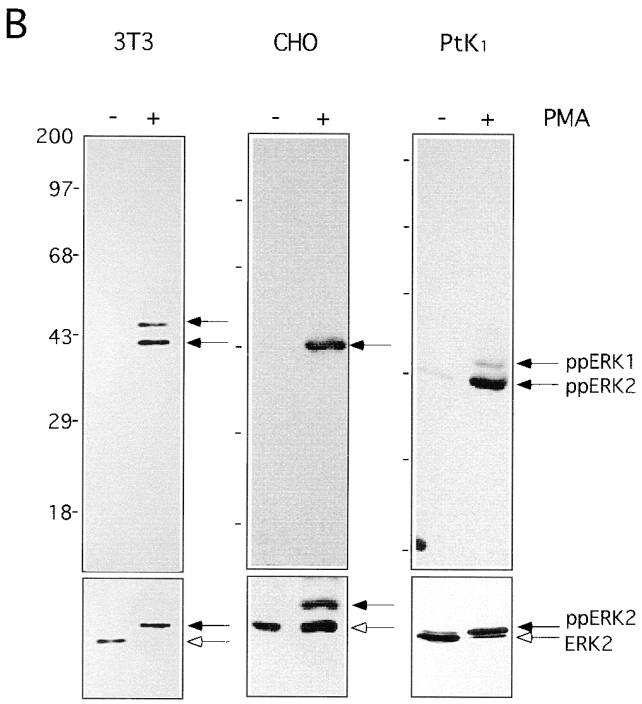
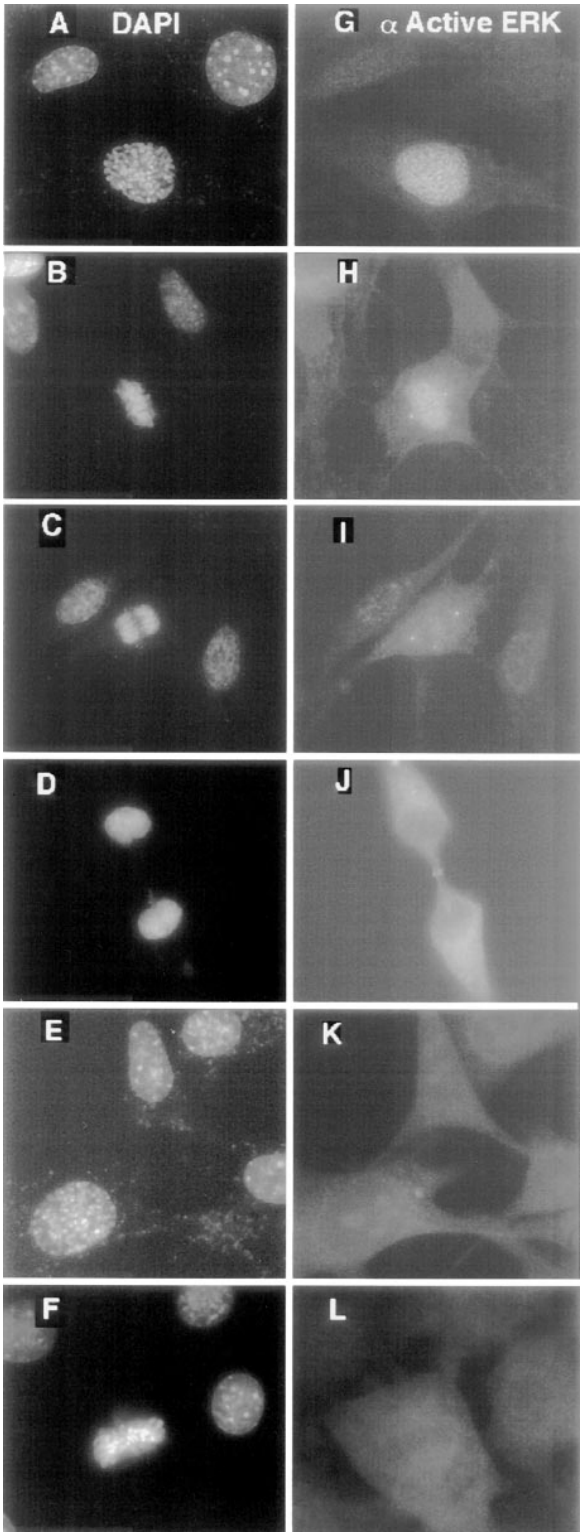


Figure 1. Specificity of anti-ACTIVE MAPK antibody. (*A*) Immunoblots of wild type or mutant recombinant ERK2 phosphorylated with constitutively active mutant MKK1-G1C. (*Top*) Immunoreactivity with anti-ACTIVE MAPK antibody. (*Bottom*) Reactivity with antibody recognizing the COOH terminus of ERK2, where ERK2 gel mobility is retarded upon phosphorylation. (*B*) Immunoblots of whole cell extracts (20 μg) from NIH 3T3, CHO, and PtK_1 cells that were serum-depleted overnight, and then treated with or without 10% serum + 0.1 μM PMA for 5 min (*PMA*). (*Top*) Specific immunoreactivity of anti-ACTIVE MAPK antibody with phosphorylated ERK1 and ERK2 (*arrows*). Only phosphorylated ERK2 was observed in CHO cell lysates. (*Bottom*) Corresponding levels of ERK2, probed with an antibody recognizing the COOH terminus of ERK2. Filled arrows show phosphorylated ERK2 with retarded mobility, and open arrows show unphosphorylated ERK2.

ERK. With phosphopeptide competition, active ERK immunoreactivity was undetectable in mitotic cells (Fig. 2, *E*, *F*, *K*, and *L*). Immunoreactivity was also absent when the primary antibody was omitted (data not shown). Experi-



(*lower cell*); (*B* and *H*) metaphase; (*C* and *I*) anaphase; and (*D* and *J*) telophase are shown. Note active ERK staining at spindle poles in metaphase and anaphase (*H* and *I*). Staining patterns were reproduced with paraformaldehyde or NBF/methanol fixation. (*E*, *F*, *K*, and *L*) In peptide competition controls, anti-ACTIVE MAPK antibody was preincubated with 0.1 mg/ml phosphopeptide before incubation with coverslips. Under these conditions, mitotic cells showed no ERK immunoreactivity, as demonstrated with prophase (*E* and *F*) and metaphase (*K* and *L*) cells.

Figure 2. Mitotic activation of ERK in NIH 3T3 cells. Cells were fixed with glutaraldehyde, and were then stained with DAPI (*A–F*) and anti-ACTIVE MAPK antibody probed with Texas Red–conjugated secondary antibody (*G–L*). Fluorescence of representative cells in (*A* and *G*) interphase (*upper cells*) and prophase

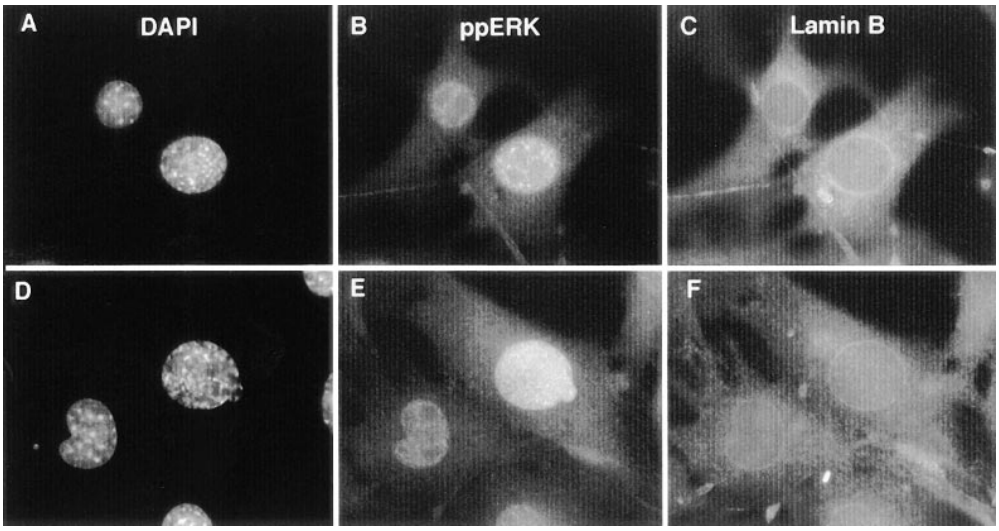


Figure 3. Activation of ERK during prophase precedes nuclear envelope breakdown. NIH 3T3 cells were fixed with glutaraldehyde, and were then stained with DAPI (*A* and *D*) and costained with (*B* and *E*) anti-ACTIVE MAPK antibodies probed with Texas Red–coupled secondary antibodies or (*C* and *F*) lamin B antibodies probed with fluorescein isothiocyanate-conjugated secondary antibodies. Patterns of lamin B staining indicate that nuclear envelope membranes are intact in prophase cells during ERK activation. Corresponding interphase cells are shown to the left of each prophase cell in the two representative images.

ments were also performed using a monoclonal antibody to active diphosphorylated ERK, and showed identical staining patterns (data not shown). Finally, experiments were performed using three different fixation protocols, including 0.1% glutaraldehyde/2% formaldehyde, 4% paraformaldehyde, or 10% neutral buffered formalin/methanol. Identical results were observed in all cases (data not shown), indicating that the cellular localization of active ERK during mitosis is not a fixation artefact.

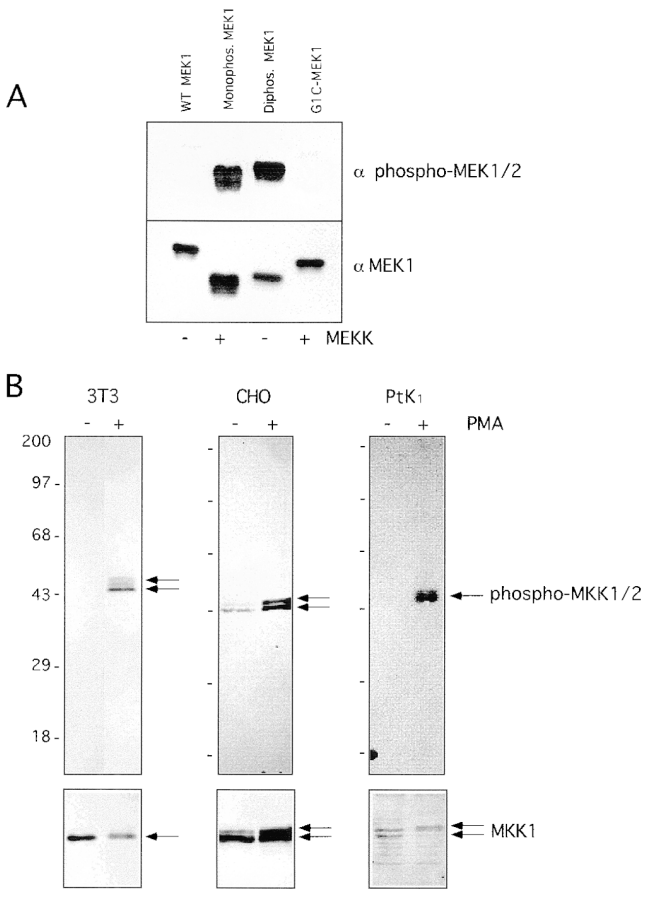


Figure 4. Specificity of anti-phosphoMEK1/2 antibody. (*A*) Immunoblots of wild-type or mutant recombinant MKK1, after phosphorylation with constitutively active mutant MEKKΔC. (*Top*) Immunoreactivity with anti-phosphoMEK1/2 antibody. (*Bottom*) Reactivity with antibody recognizing the NH_2 terminus of MKK1. Differences in gel mobility are a result of removing the 5-kD NH_2 terminal $(His)_6$ tag from mono- or diphosphorylated MKK1, and deleting residues 44–51 in MKK1-G1C. (*B*) Immunoblots of whole cell extracts (20 μg) from NIH 3T3, CHO, and PtK_1 cells that were serum-depleted overnight, and then treated with or without 10% serum + 0.1 μM PMA for 30 min. (*Top*) Specific immunoreactivity of anti-phosphoMEK1/2 antibody with phosphorylated MKK1 and MKK2, indicated by arrows. (*Bottom*) Corresponding levels of MKK1 probed with an antibody recognizing the NH_2 terminus of MKK1.

Localization of the upstream kinase, MKK, was examined using the anti-phospho-MEK1/2 antibody that is selective for active forms of MKK1 and 2. The specificity of this antibody was tested by immunoblotting MKK1 that had been phosphorylated in vitro with MEKK1 and purified by Mono-Q FPLC. This antibody reacted with both mono- and diphosphorylated MKK1, with preference for the diphosphorylated form, while no reactivity was observed with unphosphorylated MKK1 (Fig. 4 *A*, lanes *1–4*). Monophosphorylated MKK1 has measurable activity that is ~25% that of diphosphorylated enzyme (Resing et al., 1995). Therefore, this antibody distinguishes between active and inactive forms of MKK. Unlike the anti-ACTIVE ERK antibody, the anti-phospho-MEK1/2 antibody did not recognize a MKK mutant containing acidic amino acid substitutions at phosphorylation sites (G1C; Fig. 4 *A*, lane *4*). Immunoblots of whole cell extracts showed elevated reactivity with MKKs 1 and 2 after stimulation of NIH 3T3, CHO, or PtK_1 cells, but little or no reactivity with other cellular proteins (Fig. 4 *B*, *top*). Whole

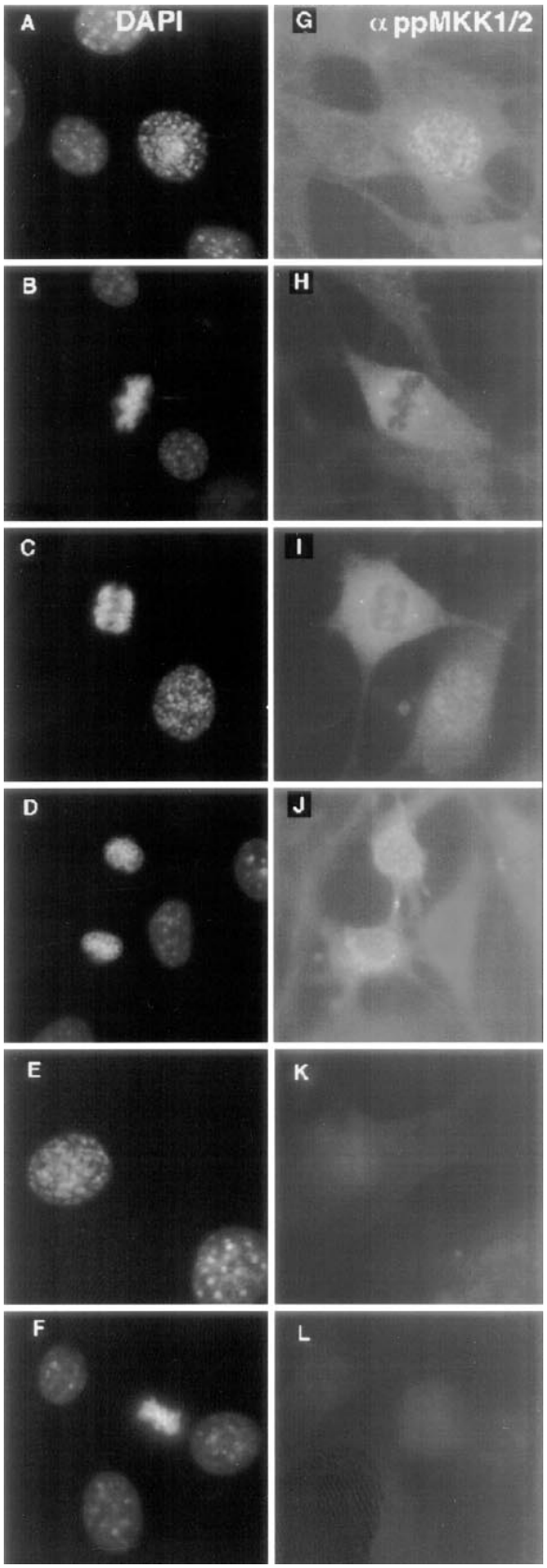


Figure 5. Mitotic activation of MKK in NIH 3T3 cells. Cells were fixed with NBF/methanol, and were then stained with DAPI (*A–F*) and anti-phosphoMEK1/2 antibody probed with Texas Red–conjugated secondary antibody (*F–L*). Fluorescence of representative cells in (*A* and *G*) interphase and prophase; (*B* and *H*) metaphase; (*C* and *I*) anaphase; and (*D* and *J*) telophase are shown. Staining patterns were reproduced with paraformaldehyde or glutaraldehyde fixation. (*E*, *F*, *K*, and *L*) In peptide competition controls, anti-phosphoMEK1/2 antibody was preincubated with 0.1 mg/ml phosphopeptide before incubation with coverslips. Under these conditions, mitotic cells showed no active MKK immunoreactivity as demonstrated with prophase (*E* and *F*) and metaphase (*K* and *L*) cells.

cell extracts were also immunoblotted using an antibody recognizing the NH_2 terminus of MKK1 to demonstrate comparable loading (Fig. 4 *B*, *bottom*).

Immunocytochemical staining of NIH 3T3 cells with the anti-phosphoMEK1/2 antibody showed little reactivity with interphase cells, but nuclear staining of active MKK increased significantly during prophase (Fig. 5, *A* and *G*). This result is notable because it represents the only case in which nuclear localization of MKK has been observed under normal cellular conditions without perturbing nuclear export (Fukuda et al., 1996; Fukuda et al., 1997; Jaaro et al., 1997). Like active ERK, localization of active MKK was apparent at spindle poles during metaphase and anaphase (Fig. 5, *B*, *C*, *H*, and *I*) and at the midbody during telophase/cytokinesis (Fig. 5, *D* and *J*). Unlike active ERK, however, active MKK appeared more diffusely localized throughout metaphase cells, and largely excluded from chromosomes (Fig. 5, *B* and *H*). Antibody preincubation with a diphosphorylated peptide corresponding to the phosphoepitope on MKK1/2 eliminated staining of mitotic cells (Fig. 5, *E*, *F*, *K*, and *L*), supporting the specificity of immunoreactivity with this antibody.

To visualize the nuclear localization of active ERK in more detail, we examined PtK_1 cells, which have fewer chromosomes and a flatter morphology during mitosis. As observed with NIH 3T3 cells, active ERK staining was absent in interphase PtK_1 cells (data not shown), but increased in nuclei during prophase (Fig. 6, *A* and *B*). Spindle pole localization of active ERK was also observed during prophase, before nuclear envelope breakdown (Fig. 6 *B*). The higher resolution available from images of PtK_1 cells showed that much of the active ERK staining was peripheral to prophase chromosomes. Furthermore, a staining pattern of paired dots could be observed on each prophase chromosome, suggesting a specific localization of active ERK to kinetochores. This pattern was confirmed by colocalization of active ERK with the microtubule motor CENP-E (Fig. 7, *C* and *D*), which localizes to kinetochores in vivo (Yen et al., 1991). Inspection of cells at different stages in prophase to prometaphase suggested a temporal order of association: active ERK appears at kinetochores earlier in prophase than CENP-E, and leaves the kinetochore earlier in metaphase/anaphase (Fig. 7). The kinetochore localization of active ERK that was so evident during prophase and prometaphase was more difficult to identify on chromosomes aligned at the metaphase plate, and was completely absent during anaphase (Fig. 6, *D* and *E,* and Fig. 7 *E*). In contrast, CENP-E staining was still apparent at kinetochores during anaphase (Fig. 7 *F*). These results suggest that either the phosphorylation or the targeting of ERK to this mechanically impor-

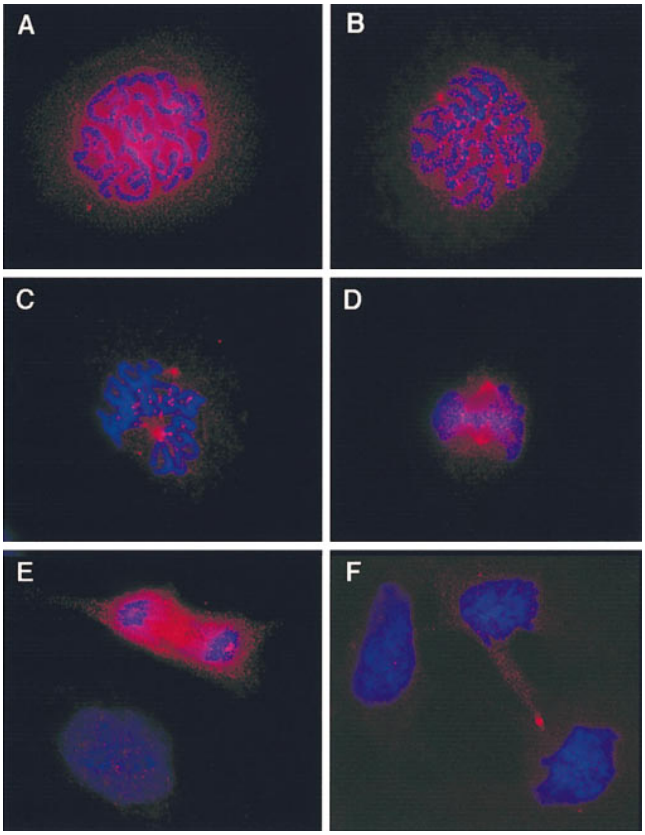


Figure 6. Mitotic activation of ERK in PtK1 cells. Cells were fixed with glutaraldehyde, and were then stained with DAPI and anti-ACTIVE MAPK antibody probed with Texas Red–conjugated secondary antibody. Fluorescence of representative cells in (*A*) early prophase; (*B*) late prophase; (*C*) prometaphase; (*D*) metaphase; (*E*) anaphase; and (*F*) telophase. Interphase cells are also shown in *E* and *F*. Staining patterns were also reproduced with paraformaldehyde or NBF/methanol fixation. Controls show no immunoreactivity when anti-ACTIVE MAPK antibody was preincubated with 0.1 mg/ml phosphopeptide before incubation with coverslips (data not shown).

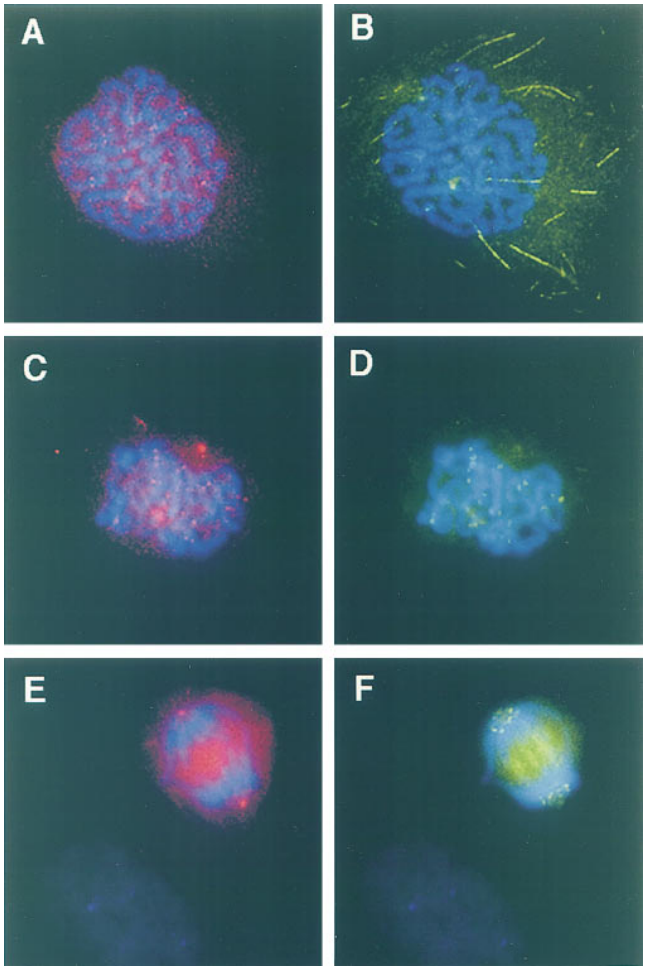


Figure 7. Active ERK and CENP/E colocalize at kinetochores. PtK_1 cells were fixed with glutaraldehyde, and were then stained with DAPI together with mouse monoclonal anti-phosphoMAPK antibody and rabbit anti-CENP-E antibody, probed with Texas Red– or fluorescein isothiocyanate–conjugated secondary antibodies, respectively. Fluorescence of representative cells in (*A* and *B*) prophase; (*C* and *D*) prometaphase; and (*E* and *F*) anaphase; showing kinetochore staining of active ERK early in prophase, before the appearance of CENP-E, and disappearance of kinetochore-associated active ERK during anaphase before the disappearance of CENP-E. An interphase cell is also shown in *E* and *F*.

tant region decreases as chromosomes separate. With phosphopeptide competition, active ERK immunoreactivity was undetectable in mitotic PtK_1 cells (data not shown).

PtK_1 staining was also examined using an antibody recognizing the COOH terminus of ERK2, which is not selective for the protein's state of phosphorylation. An abundance of total ERK2 was observed throughout PtK_1 cells. In interphase cells, ERK was primarily localized in cytoplasmic pools as previously reported (Lenormand et al., 1993), but in prophase, nuclear staining increased significantly, indicating the relocalization of a significant fraction of the total ERK pool (Fig. 8). However, in contrast to active ERK, kinetochore localization within the total pool of nuclear ERK was masked, indicating that the active enzyme associating with kinetochores represents a small fraction of total ERK.

Although kinetochore staining of active ERK was not apparent in fixed and permeabilized NIH 3T3 cells (Fig. 2), we were also unable to observe CENP-E staining, indicating that kinetochores were refractory to staining in these experiments, perhaps due to cell shape. Consistent with this finding, colocalization of active ERK and CENP-E at kinetochores was observed in porcine kidney epithelial $LLCPK_1$ cells, which also exhibit a flat morphology during mitosis (data not shown). As noted below, kinetochore localization of active ERK was also observed on isolated chromosomes from NIH 3T3 cells, CHO cells, and 9948 human lymphoblasts. Active ERK localized to kinetochores of every cell type we examined, indicating that it represents a general phenomenon in mammalian cells.

Examination of phospho-MKK in PtK_1 cells also showed increased nuclear staining during prophase, with spindle pole localization during prophase to anaphase, and was mostly excluded from chromosome arms (Fig. 9, *A–D*).

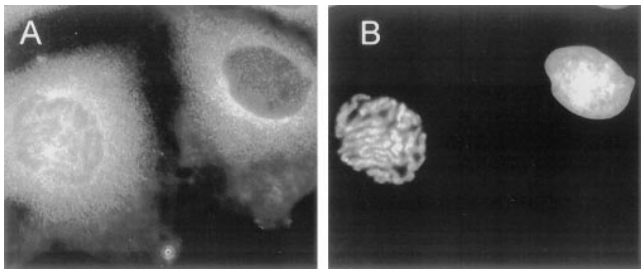


Figure 8. Active ERK localized to kinetochores is a minor fraction of the total ERK pool. PtK_1 cells were fixed with glutaraldehyde, and were then stained with (*A*) COOH-terminal ERK2 antibody probed with Texas Red–conjugated secondary antibody; or (*B*) DAPI. Fluorescence of representative cells in prophase and interphase are shown in each panel. Although a significant percentage of ERK is nuclear in prophase, kinetochore localization cannot be detected.

In general, phospho-MKK showed infrequent reactivity with kinetochores compared with phospho-ERK, although paired dot patterns were evident in some cases (Fig. 9 *B*). These data indicate that active ERK and active MKK accumulate in nuclei during prophase, and suggest that active ERK might preferentially associate with kinetochores. These results suggest that ERK phosphorylation might serve as a signal for chromosome targeting.

Indeed, the temporal pattern of phosphoERK association with kinetochores is similar to that observed using 3F3/2, a monoclonal antibody raised to crude maturation promoting factor (MPF) preparations that recognizes nuclear phosphorylated antigens (Cyert et al., 1988; Gorbsky and Ricketts, 1993). Kinetochore phosphoepitopes have been shown to react with this antibody under conditions where chromosomes are misaligned or detached from microtubules, suggesting that the decreases in antigen reactivity that occur during mitotic progression are caused by dephosphorylation as the chromosomes align under microtubule-induced tension at the metaphase plate (Gorbsky and Ricketts, 1993; Nicklas et al., 1995).

Temporal correlations in mitotic localizations of active ERK and the 3F3/2 phosphoepitope were observed by double immunofluorescence staining of PtK1 cells. Kinetochore colocalization of both antigens was apparent from early prophase through prometaphase (Fig. 10, *A–D*). In metaphase cells, partial kinetochore staining could sometimes be observed with anti-ACTIVE MAPK antibody, but never with 3F3/2 antibody (Fig. 10, *E* and *F*), suggesting that the dephosphorylation of the 3F3/2 antigen during metaphase precedes inactivation of ERK. In both cases, kinetochore staining was absent on chromosomes in anaphase (data not shown). The dependence of kineto-

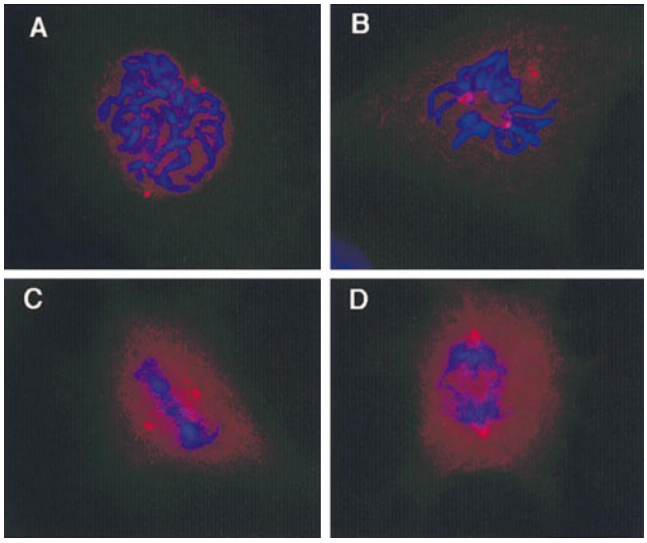


Figure 9. Mitotic activation of MKK in PtK1 cells. Cells were fixed with glutaraldehyde, and were then stained with DAPI and anti-phosphoMEK1/2 antibody probed with Texas Red–conjugated secondary antibody. Fluorescence of representative cells in (*A*) prophase; (*B*) prometaphase; (*C*) metaphase; and (*D*) anaphase. Staining patterns were reproduced with paraformaldehyde or NBF/methanol fixation. Controls show no immunoreactivity when anti-phosphoMEK1/2 antibody was preincubated with 0.1 mg/ml phosphopeptide before incubation with coverslips (data not shown).

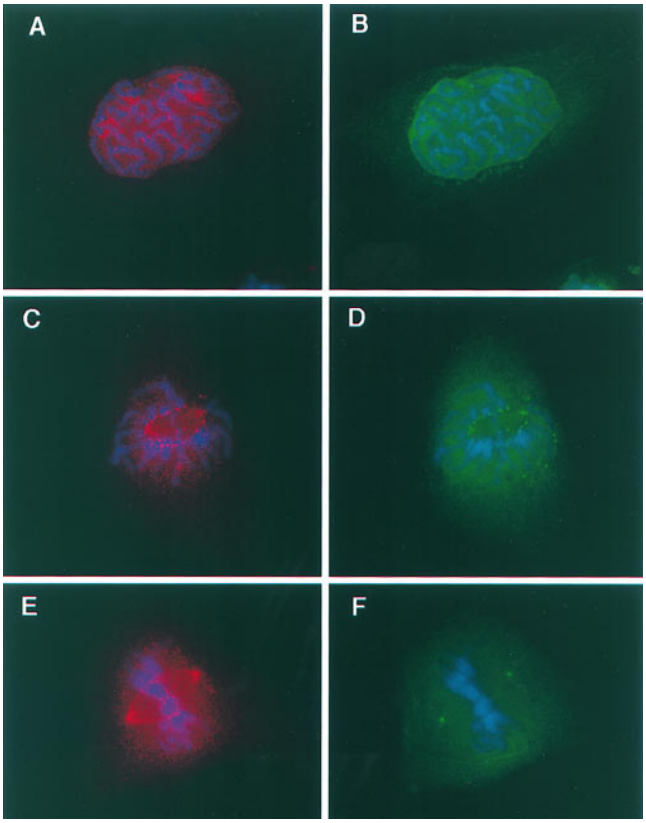


Figure 10. Active ERK and 3F3/2 antigen colocalize at kinetochores. PtK_1 cells were fixed with glutaraldehyde, and were then stained with DAPI, together with anti-ACTIVE MAPK antibody probed with Texas Red–coupled secondary antibody and 3F3/2 antibody probed with fluorescein isothiocyanate-conjugated secondary antibody. Fluorescence of representative cells in (*A* and *B*) prophase; (*C* and *D*) prometaphase; and (*E* and *F*) metaphase, showing corresponding appearances of active ERK and 3F3/2 antigen at kinetochores early in prophase, and disappearance at or after metaphase.

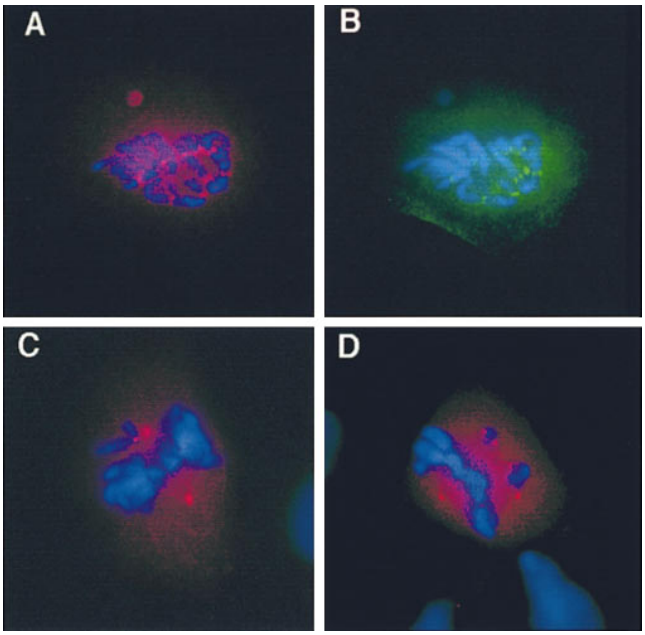


Figure 11. Kinetochore localization of active ERK correlates with microtubule disruption or chromosome displacement. (*A* and *B*) PtK_1 cells were treated with nocodazole for 30 min, and were then fixed with glutaraldehyde and stained with DAPI, anti-ACTIVE MAPK antibody, and 3F3/2 antibody as in Fig. 10. Fluorescence of representative cells in metaphase showed stable kinetochore staining of (*A*) active ERK and (*B*) 3F3/2 antigen. (*C* and *D*) Untreated PtK_1 cells fixed and stained with DAPI or anti-ACTIVE MAPK antibody. High immunoreactivity of active ERK is observed on metaphase chromosomes that are misaligned from the metaphase plate.

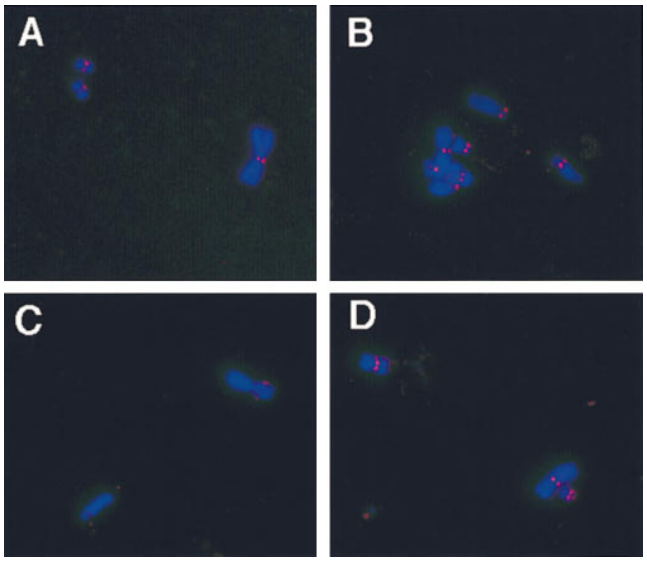


Figure 12. Kinetochore localization of active ERK and 3F3/2 antigen on isolated chromosomes. (*A* and *B*) Isolated CHO cell chromosomes bound to glass coverslips were stained without fixation with DAPI and (*A*) anti-ACTIVE MAPK antibody or (*B*) 3F3/2 antibody, probed in each case with Texas Red–conjugated secondary antibodies. (*C* and *D*) Isolated chromosomes were incubated with added (*C*) PP2A for 15 min, or (*D*) PP2A for 15 min, followed by 2 μM microcystin, 2 μg active ERK2, and MgATP for 10 min. Chromosomes were then stained with DAPI and 3F3/2 antibody, followed immediately by data collection.

chore localization of active ERK on microtubule attachment was also examined. Treatment of cells with nocodazole to disrupt spindle microtubules resulted in stabilized kinetochore localization of active ERK as well as the 3F3/2 antigen (Fig. 11, *A* and *B*). Untreated metaphase cells were inspected for chromosomes that were aberrantly displaced from the metaphase plate, which is easily identifiable by DAPI staining. In several such examples, kinetochores on displaced chromosomes showed significantly enhanced staining with anti-ACTIVE MAPK antibody compared with the reduced staining of aligned chromosomes (Fig. 11, *C* and *D*). Thus, the activity of ERKs bound to kinetochores appears to correlate inversely with kinetochore-microtubule attachments, consistent with the behavior of the 3F3/2 phosphoantigen, as reported previously (Gorbsky and Ricketts, 1993; Nicklas et al., 1995).

To explore a possible functional relationship between active ERK and the 3F3/2 antigen, chromosomes were isolated from CHO cells, immobilized on coverslips within perfusion chambers, and visualized by immunofluorescence. Both active ERK and 3F3/2 reactive antigens were observed at kinetochores (Fig. 12, *A* and *B*). Staining on chromosome arms was visible, but low compared with kinetochore staining, indicating that the most stable association is at the kinetochores. Similar results were observed with isolated chromosomes from NIH 3T3 cells or 9948 lymphoblasts (data not shown). After perfusion with PP2A, the fluorescence staining for 3F3/2 antigens at kinetochores of CHO cells was dramatically decreased (Fig. 12 *C*). At least some of this activity returned after treating phosphatase-treated chromosomes with microcystin, active diphosphorylated ERK2, and Mg/ATP (Fig. 12 *D*).

These results motivated an optical quantification of epitope levels after various experimental treatments. Quantitative measurements showed decreased staining of kinetochores with anti-ACTIVE MAPK antibody in response to phosphatase treatment (Fig. 13 *A*), conditions where dephosphorylation of ERK would be expected. In addition, the fluorescence intensity of 3F3/2 antibody staining at kinetochores decreased by 75% after 15 min of reaction with PP2A (Fig. 13, *B* and *C*), as compared with controls in which PP2A was omitted or dephosphorylation was blocked with microcystin (Fig. 13, *B* and *C*). This result is consistent with the proposed recognition of a phosphorylated epitope by the 3F3/2 antibody (Cyert et al., 1988; Gorbsky and Ricketts, 1993).

After PP2A treatment, microcystin was added to block dephosphorylation, and purified diphosphorylated ERK2 was perfused into chambers along with MgATP. In this case, the fluorescence intensity of 3F3/2 staining increased by four to fivefold after 10 min of reaction with wild-type active ERK2 (Fig. 13, *B* and *C*). The staining also increased by about twofold when chromosomes were perfused with MgATP alone, or when perfused with MgATP in presence of diphosphorylated, catalytically inactive mutant ERK2-K52R. Furthermore, perfusion of chromosomes with constitutively active mutant MKK1-G7B in the

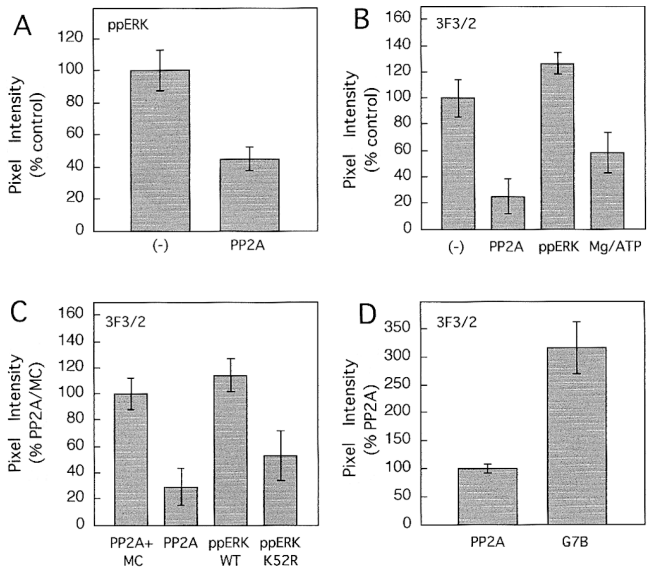


Figure 13. Sensitivity of active ERK or 3F3/2 antigen at isolated kinetochores to dephosphorylation or rephosphorylation by added enzymes. (*A*) Isolated chromosome samples bound to glass coverslips were incubated without or with PP2A (2U) for 15 min, followed by staining with DAPI and anti-ACTIVE MAPK antibody, as in Fig. 12. 8–14 kinetochores were examined for each condition. (*B*) Chromosomes were incubated without or with PP2A for 15 min, with PP2A followed by 2 μM microcystin, 2 μg active ERK2, and MgATP, or with PP2A followed by microcystin and MgATP for 10 min. 12–18 kinetochores were examined for each condition. (*C*) Chromosomes were incubated with PP2A+microcystin for 15 min, with PP2A for 15 min minus inhibitor, with PP2A for 15 min followed by microcystin, active ERK2, and MgATP, or with PP2A for 15 min followed by microcystin, diphosphorylated catalytically inactive ERK2-K52R, and MgATP. 6–9 kinetochores were examined for each condition. (*D*) Chromosomes were incubated with PP2A for 15 min minus inhibitor followed by microcystin and MgATP or microcystin, active MKK1-G7B, and MgATP for 15 min. Eight kinetochores were examined for each condition. Average fluorescence intensities and standard errors are shown, normalized to controls in each experiment.

presence of MgATP after PP2A treatment led to a significant increase in 3F3/2 staining at kinetochores compared with perfusion with MgATP alone (Fig. 13 *D*).

Immunoblotting of active ERK showed no reactivity with 3F3/2 antibody (data not shown), indicating that diphosphorylated ERK is not likely to be the 3F3/2 reactive phosphoantigen. Taken together, our results are consistent with a model in which the 3F3/2 antigen can be phosphorylated in response to addition of exogeneous ERK2, as well as by endogenous protein kinases located at kinetochores, possibly including ERK2. These findings indicate that the 3F3/2 phosphoepitope may be regulated by ERK, suggesting a role for ERK function at kinetochores in mediating responses to the microtubule attachments that control anaphase onset.

Discussion

The results presented above suggest that active forms of both ERK and MKK become concentrated in the prophase nuclei of somatic mammalian cells. ERK is localized particularly to the kinetochores of the condensing chromosomes. Centrosomal staining is also seen with both antigens. It becomes clear at prometaphase and is maintained through anaphase, but the strength of ERK localization at kinetochores decreases as the chromosomes attach to the spindle and become organized at the metaphase plate. This behavior is reminiscent of the staining seen with 3F3/2, a monoclonal antibody that recognizes a phosphoepitope found on mature but unattached chromosomes. The intensity of 3F3/2 staining of kinetochores on isolated mammalian chromosomes treated with phosphatases and kinases is consistent with the hypothesis that ERK contributes to the formation of the 3F3/2 epitope, and therefore may play a role in regulating kinetochore function during mitosis.

Our results with antibodies selective for the active phosphorylated forms of ERK and MKK demonstrate significant increases in the specific localizations of the endogeneous enzymes during mammalian cell mitosis. The concentration of these enzymes in nuclei during early prophase of cycling somatic cells supports a role for the MAP kinase pathway in regulating early mitotic events. This result was unexpected because previous biochemical studies of kinase activity measured by SDS-PAGE gel mobility retardation or in-gel phosphorylation assays of mammalian cell extracts failed to show ERK activation during mitosis (Tamemoto et al., 1992; Edelmann et al., 1996). These differences can be reconciled by comparing the staining patterns seen with the anti-ACTIVE MAPK antibody versus staining with an antibody recognizing both unphosphorylated and phosphorylated ERK. The mitotically active pool of ERK appearing at discrete mitotic locations represents a minor fraction of the total ERK, and thus may not be observable by examining bulk kinase activity. This highlights the key advantage of antibodies that selectively recognize active phosphorylated forms of regulatable enzymes for studies of cell process control.

A requirement for ERK in M phase entry has been extensively studied in several model systems for meiotic cell division (reviewed by Yew et al., 1994; Sagata, 1997). During oocyte maturation, ERK activation promotes release from G2 arrest and entry into metaphase I. In dividing somatic cells, activation of ERK by active mutant MKK expression enhances cell growth rate, consistent with a role in promoting cell cycle progression (Seger et al., 1994). Significantly, recent findings suggest a requirement for MKK and ERK during the G2-M transition in cycling mammalian cells (J.H. Wright and E.G. Krebs, personal communication). In these studies, synchronized NIH 3T3 cells allowed to progress through S phase were retarded in M phase entry after inhibition of MKK/ERK with a dominant negative mutant MKK1 or the MKK1 inhibitor, PD98059. Under these conditions, tyrosine dephosphorylation of cdc2 was suppressed, indicating that MKK/ERK is somehow involved in mitotic entry upstream of cyclin B/cdc2 activation. These findings are consistent with our results showing that MKK and ERK are activated early in prophase before nuclear envelope breakdown.

ERK also prevents release from metaphase II arrest in oocytes through inhibition of cyclin B degradation and sta-

bilization of cdc2 activity (Sagata, 1997). Injection of Mos, active MKK, or active ERK into two-cell frog embryos induces a similar M phase arrest with elevated cyclin B/cdc2 activity (Haccard et al., 1993; Kosako et al., 1994). Similar findings were reported in frog embryo extracts, where G2 arrest as well as M phase arrest were observed in response to Mos, MKK, or ERK, depending on the timing of addition (Abrieu et al., 1997; Walter et al., 1997). These results may be important in understanding ERK and mechanisms for spindle checkpoint regulation, given that mitotic frog extracts undergo metaphase arrest when challenged with high levels of sperm DNA together with microtubule-destabilizing agents in a manner that depends upon elevated ERK activity and stabilization of cyclin B/cdc2 activity (Minshull et al., 1994; Takenaka et al., 1997). These data suggest that ERK promotes metaphase spindle checkpoint arrest by interfering with the activation of the anaphase-promoting complex (APC), perhaps by functioning upstream or in parallel with other checkpoint regulatory pathways (Abrieu et al., 1996).

The kinetochore localization of active ERK that we observe in PtK_1 cells suggests a mechanism by which ERK might regulate anaphase entry. Previous findings indicate that microtubule tension on chromosomes and kinetochore attachment is an important feature of the mitotic checkpoint regulating anaphase entry (reviewed by Nicklas, 1997). In particular, it has been suggested that dominant signals derived from unattached kinetochores may delay anaphase entry (McIntosh, 1991). Such signals may involve phosphorylation of proteins within pathways that regulate APC activation and cyclin B degradation, which are required for the metaphase-to-anaphase transition (Gorbsky, 1997; Nigg et al., 1996). A useful probe for phosphorylation signals has been the 3F3/2 monoclonal antibody, originally raised to impure MPF preparations, which appears to recognize phosphorylated antigens located at kinetochores. Reactivity with kinetochores is high during prophase and prometaphase, and appears to diminish during metaphase as microtubules attach. On the other hand, reactivity increases during kinetochore detachment, and loss of tension induced in response to nocodazole or by physical misalignment of chromosomes away from the metaphase plate (Gorbsky and Ricketts, 1993; Nicklas et al., 1995). Furthermore, 3F3/2 antibody microinjection into cycling cells delays anaphase onset, suggesting that dephosphorylation of this antigen is a necessary event for chromosome segregation (Campbell and Gorbsky, 1995).

We observed that the appearance and disappearance of active ERK at kinetochores resembled the timing and behavior of 3F3/2 immunoreactivity previously reported, suggesting that regulation of ERK may be linked somehow to 3F3/2 antigen phosphorylation. Active ERK appeared at kinetochores during prophase and prometaphase and disappeared at some point during the metaphase-to-anaphase transition. Furthermore, stable kinetochore staining was observed under conditions of microtubule disruption or on chromosomes that were displaced from the metaphase plate. Isolated chromosomes provided an excellent model system to test whether kinetochore 3F3/2 antigens could be modulated by ERK activity, allowing quantification of immunostaining after direct reactions with added enzymes. Interestingly, both active ERK and the 3F3/2 antigen were present on kinetochores, indicating a stable association of these antigens with isolated chromosomes. Reduced staining of each was observed after protein phosphatase treatment, most likely due to phosphoepitope dephosphorylation. Importantly, the enhanced reactivity of kinetochores with 3F3/2 antibody after incubation with active ERK2 or active MKK1 indicates that 3F3/2 antigen(s) are likely to be targets for ERK or ERK-regulated kinases. The only known substrates for MKK1 are ERKs 1 and 2, suggesting that the 3F3/2 antigen is phosphorylated by ERK or an ERK-regulated kinase, endogeneously localized at kinetochores. It is therefore reasonable that ERK in its active form might serve as a signal at kinetochores, allowing cells to sense improper chromosome attachment to spindle microtubules via phosphorylation of the 3F3/2 antigen. Dephosphorylation of the 3F3/2 antigen after microtubule attachment and resulting increased tension at kinetochores might then release the inhibitory checkpoint machinery, facilitating APC activation and protein degradation events needed for anaphase entry.

Other MAP kinase signaling pathways may also be involved in M-phase progression. The p38 MAPK has been implicated in spindle assembly checkpoint and mitotic arrest in mammalian cells (Takenaka et al., 1998), based on data reporting the activation of p38 MAPK during nocodazole-induced mitotic arrest and partial suppression of the nocodazole-induced checkpoint by a p38 MAPK inhibitor. So far we have found no evidence for p38 MAPK activation in mitotic cells, localization of active enzyme at kinetochores of permeabilized cells, or isolated chromosomes using an antibody from New England Biolabs (Beverly, MA) that recognizes active, diphosphorylated p38 MAPK (data not shown). The specificity of this antibody was confirmed by enhanced immunofluorescence of permeabilized cells or reactivity in immunoblots of cells exposed to UV irradiation (data not shown). Therefore, p38 MAPK is probably not involved in 3F3/2 antigen phosphorylation.

Another important result from our study is the localization of active ERK at the chromosome periphery during prophase and prometaphase, as well as at spindle poles during prophase, metaphase, and anaphase. The pattern of active ERK on spindle microtubules, spindle poles, and kinetochores suggests a role of ERK in regulating spindle assembly, possibly through regulation of motor activity or microtubule polymerization. Spindle pole immunostaining was previously reported during meiotic metaphase in mouse oocytes and mitosis in *Xenopus* tadpole cells (Verlhac et al., 1993; Wang et al., 1997). ERK staining of microtubules has also been observed in NIH 3T3 cells, though it is somewhat dependent on the antibody used (Rezka et al., 1995), and association of active and inactive ERK pools with tubulin and MAPs has been demonstrated biochemically (Morishima-Kawashima and Kosik, 1996; Mandelkow et al., 1992). Furthermore, adding active MAPK to interphase frog embryo extracts leads to microtubule shortening and organization, which resembles a mitotic array (Gotoh et al., 1991). Potential mediators of microtubule depolymerization include MAPs such as tau, which, upon phosphorylation by ERK enhances the probability of microtubule depolymerization (Shiina et al., 1992;

Hoshi et al., 1992; Dreschsel et al., 1992). Another possible target is stathmin/Op18, which facilitates microtubule catastrophe and mitotic destabilization of interphase microtubules (Belmont and Mitchison, 1996), and is hyperphosphorylated during mitosis at sites targeted by several kinases, including ERK (Brattsand et al., 1994; Marklund et al., 1993; Larsson et al., 1995).

In summary, current data suggest that the MKK/ERK pathway plays multiple roles during mitosis, promoting critical events that regulate mitotic entry or spindle assembly and function, as well as inhibiting events needed for anaphase entry. Our findings raise new questions about downstream targets for ERK at different stages of mitotic progression, and the potential overlap in specificity between ERK and cyclin B/cdc2 for substrates. Further questions are also raised regarding the mechanism of MKK and ERK activation in G2. It is reasonable to suggest that this regulatory pathway involves at least some of the components identified as upstream regulators in G1. The mitotic activation of MKK and its requirement for mitotic entry in NIH 3T3 cells (J.H. Wright and E.G. Krebs, personal communication) is consistent with previous reports of mitotic activation of Src and Raf as well as a requirement for Src activation during M phase entry (Roche et al., 1995; Laird et al., 1995). At this time, no evidence exists for a mitotic role for Ras that may explain Src or Raf regulation. Therefore, the possibility exists that alternative mechanisms, perhaps involving novel upstream regulators of the MAPK pathway, function to control M phase entry. Finally, an important event revealed in our studies is the nuclear uptake of both MKK and ERK during prophase, before nuclear envelope breakdown. Although previous work has demonstrated ERK translocation after activation in response to growth factors (Lenormand, 1993), nuclear localization of MKK has been demonstrated only with MKK mutants lacking its nuclear export signal or under conditions where nuclear export is inhibited (Fukuda, 1996; Fukuda, 1997; Jaaro et al., 1997). Localization of active MKK during prophase identifies a unique physiological condition where nuclear uptake is favored, and specifies an important function of regulatory mechanisms for nuclear import during cell cycle progression.

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