Tyrosine-phosphorylated Extracellular Signal–regulated Kinase Associates with the Golgi Complex during G2/M Phase of the Cell Cycle: Evidence for Regulation of Golgi Structure

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Abstract. Phosphorylation of the extracellular signal–regulated kinases (ERKs) on tyrosine and threonine residues within the TEY tripeptide motif induces ERK activation and targeting of substrates. Although it is recognized that phosphorylation of both residues is required for ERK activation, it is not known if a single phosphorylation of either residue regulates physiological functions. In light of recent evidence indicating that ERK proteins regulate substrate function in the absence of ERK enzymatic activity, we have begun to examine functional roles for partially phosphorylated forms of ERK. Using phosphorylation site–specific ERK antibodies and immunofluorescence, we demonstrate that ERK phosphorylated on the tyrosine residue (pY ERK) within the TEY activation sequence is found constitutively in the nucleus, and localizes to the Golgi complex of cells that are in late G2 or early mitosis of the cell cycle. As cells progress through metaphase and anaphase, pY ERK localization to Golgi vesicles is most evident around the mitotic spindle poles. During telophase, pY ERK associates with newly formed Golgi vesicles but is not found on there after cytokinesis and entry into G1. Increased ERK phosphorylation causes punctate distribution of several Golgi proteins, indicating disruption of the Golgi structure. This observation is reversible by overexpression of a tyrosine phosphorylation–defective ERK mutant, but not by a kinase-inactive ERK2 mutant that is tyrosine phosphorylated. These data provide the first evidence that pY ERK and not ERK kinase activity regulates Golgi structure and may be involved in mitotic Golgi fragmentation and reformation.

Key words: mitosis • Golgi complex • tyrosine phosphorylation • MAP kinase • cell cycle

Introduction

Extracellular signal–regulated kinase (ERK)[1] activity is regulated by mitogen-activated protein kinase (MAPK)/ERK kinase1/2 through phosphorylation on tyrosine (Y_{185} [numbering according to mouse ERK2 sequence]; Swiss-prot:P27703) and then threonine (T_{183}), and both phosphorylations are required for ERK activation (Seger et al., 1992). Although it is generally accepted that ERK activation and substrate phosphorylation are the primary means by which ERK regulates protein function, recent data support a function for ERK proteins that is not dependent on phosphorylation and enzymatic activity. For example, un-

phosphorylated yeast MAPK (Kss1) can repress transcription activity through direct interactions with the Ste12 transcription factor and the nuclear proteins Dig1 and Dig2 (Bardwell et al., 1998a,b). After activation by Ste7 MAPK kinase (MKK), the phosphorylated Kss1 can no longer form the repressor complex and transcription can ensue. In mammalian cells, the phosphatase activity of the dual-specificity phosphatase MAPK phosphatase 3 (MKP-3) can be enhanced by nonactive ERK2 interactions with its NH_2-terminal region (Camps et al., 1998). Additionally, ERK2 interactions with the DNA-modifying enzyme topoisomerase IIα (Topo IIα) can enhance the ability of Topo IIα to relax or decatenate DNA through a mechanism that is dependent on the phosphorylation status of ERK2, but not ERK2 activity (Shapiro et al., 1999). Therefore, in addition to their roles as kinases, ERKs likely possess the ability to modulate target protein function in a manner that does not require enzymatic activity.

It is not known whether partially phosphorylated forms of ERK, including monophosphorylations on either the tyrosine or threonine residue within the activating site, play

a functional role in regulating protein targets. Antibodies have been developed against phosphotyrosine ERK (pY ERK), phosphothreonine ERK (pT ERK), or dually phosphorylated ERK (pTpY ERK), and are useful tools for studying the mechanisms of ERK activation and inactivation after growth factor stimulation (Yao et al., 2000). These studies suggest that the phosphorylation status of ERK is dependent on the balance between ERK kinase and phosphatase activities, which is determined by the subcellular location of ERK and the stimulus.

The ERK pathway is an important mediator of extracellular signals that promote cell growth. ERK proteins regulate cell growth by promoting cell cycle transitions as cells exit quiescence and progress through G1 into S phase. ERK activity during G1 transitions is required for the phosphorylation and activation of transcription factors, induction of immediate early genes, enhanced cyclin D1 expression, and activation of translation factors (Beno et al., 1995; Pelech and Charest, 1995; Lavoie et al., 1996). In contrast, the role of the ERK pathway in regulating other phases of the somatic cell cycle and, in particular, its role during mitotic entry and exit, has not been clearly defined. In the absence of stable MKK or ERK activation, activation of Raf-1 during mitosis has been reported through a mechanism that is not dependent on Ras or typical growth factor receptor coupling (Laird et al., 1999). However, the targets or function of Raf-1 during mitosis are not known. In addition, Src and Src-like proteins, which are involved in Raf-1 and ERK pathway activation, appear to be important mediators of G2 transition mouse fibroblasts (Roche et al., 1995). Events downstream of Raf-1 may also function during G2/M transitions. Stable expression of nonactivatable MKK1 in mouse fibroblasts has been reported to delay G2 transition into mitosis (Wright et al., 1999). Similarly, the loss of MKK2 inhibits the ability of cells to recover from G2 arrest induced by ionizing radiation, supporting a role for MKK2 in G2 progression (Abbott and Holt, 1999). These examples illustrate the fact that a role for ERK activity during G2/M has not been established, suggesting either that MKK proteins have alternative substrates, or that ERK's involvement is through a mechanism that does not require ERK activation.

In previous studies, we have reported that both MKK and ERK proteins are activated early in mitosis, and that ERK may modulate the phosphorylation of kinetochore proteins that are essential for metaphase to anaphase transitions (Shapiro et al., 1998; Tolwinski et al., 1999). Centromere protein E (CENP-E), an essential kinetochore protein, is a potential target that interacts with, and is phosphorylated by, ERK2 (Zecevic et al., 1998). However, the functional significance of ERK phosphorylation of CENP-E has not been determined. Furthermore, inhibition of MKK with chemical inhibitors supported a role for the Raf-1/MKK/ERK pathway in regulating the accumulation of mitotic cells after nocodazole treatment (Hayne et al., 2000). Recently, the ERK pathway has been implicated in regulating Golgi fragmentation during mitosis. During early mitosis, the Golgi complex disassembles, such that Golgi components can be distributed equally to each daughter cell and reassembled upon completion of mitosis. The interphase Golgi network, comprised of flattened stacks of cisternae, fragments into smaller stacks

and eventually forms clusters of vesicles and tubules during mitosis (Levine et al., 1995; Shima et al., 1997). Golgi fragmentation during mitosis is regulated by protein phosphorylation, and may involve activation of an ERK1/2 activating kinase, MKK1 (Acharya et al., 1998; Colanzi et al., 2000). Although this effect could be ascribed to MKK1 activation, there was no evidence that ERK1/2 protein activation was involved in this process. Nonetheless, the presence of an ERK-like protein on Golgi complexes was suggested (Acharya et al., 1998). In contrast, others report that the cell cycle–dependent protein kinase Cdc2, and not MKK1, is the major regulator of mitotic Golgi fragmentation (Lowe et al., 1998; Draviam et al., 2001). A proposed mechanism for fragmentation may involve phosphorylation of Golgi structural protein GM130 at serine 25 by Cdc2, which disrupts interactions with the vesicle-docking protein p115 (Lowe et al., 1998). Further studies have demonstrated that as cells transition through mitosis, the timing of GM130 phosphorylation and dephosphorylation correlated with the dissociation and reassociation of p115 and Golgi fragmentation (Lowe et al., 2000). In support of both Cdc2 and MKK1, Kano et al. (2000) report that MKK1 and Cdc2 are temporally involved in mitotic Golgi fragmentation. These studies suggested that MKK1 is involved in the initial phases of Golgi fragmentation in which the stacks of cisternae are broken up into ministacks, whereas Cdc2 is responsible for later stages of fragmentation in which the Golgi complex is further broken up and vesicle fragments are more thoroughly distributed throughout the cytoplasm.

In this work we have examined the phosphorylation status of ERK proteins in cells, using phosphospecific ERK antibodies in order to examine ERK regulation and function during the cell cycle. We show that pY ERK associates transiently with Golgi membranes and vesicles during entry and exit from mitosis, and that it may regulate the Golgi structure. These findings suggest that partially phosphorylated forms of ERK regulate the dynamics of subcellular structures during mitosis, and that ERK proteins can affect substrate function through mechanisms independent of kinase activity.

Materials and Methods

Reagents and Materials

Stock solutions of nocodazole (5 mg/ml; Sigma-Aldrich) or taxol (4 mM; Sigma-Aldrich) in DMSO were diluted to a final concentration of 100 ng/ml and 1 μM, respectively. Lamin B (M-20), pY ERK (E-4), ERK1 (C-16), ERK2 (C-14), and MKK1 (C-18) antibodies were purchased from Santa Cruz Biotechnology, Inc. Other antibodies used include antiphospho-MKK1/2 (pS217, pS221; New England Biolabs, Inc.), and anti–pY ERK (M3682), pTpY ERK (M8159), and α-tubulin (Sigma-Aldrich). Golgi antibodies against p115 and GM130 (BD PharMingen/Transduction Labs) used include Rab6 (Santa Cruz Biotechnology, Inc.), p97 Golgin (Molecular Probes), and mannosidase II (Mann II) (gift of Dr. Marilyn Farquhar, University of California, San Diego, La Jolla, CA). Tyrosine-phosphorylated and unphosphorylated ERK peptides were synthesized in the University of Maryland Biopolymer Laboratory using the amino acid residues HTG-FLTE(p)Y$_{185}$VAT on ERK2, which corresponded to the sequence recognized by the monoclonal pY ERK–specific antibody (Yao et al., 2000).

ERK2 Mutagenesis

Using ERK2 wild-type cDNA as a template, ERK2 tyrosine to phenylalanine (ERK2 TEF) mutant was generated by PCR with the following

primer sets: (a) forward 5′-GAG<u>AAGCTT</u>ATGGCGGCGGCGGCG-3′ (HindIII site is underlined) and reverse mutagenic 5′-TGTGGCCA-CAAATTCTGTCAGGAACCC-3′, and (b) reverse 5′-TAT<u>CTCGAGT</u>-TAAGATCTGTATCC-3′ (XhoI site is underlined) and forward mutagenic 5′-CTGACAGAATTTGTGGCCACACGT-3′. ERK2 TEF was subcloned into the HindIII-XhoI site of pCDNA3 (Invitrogen), verified by sequencing, and tyrosine-defective phosphorylation was confirmed by the inability to be recognized by the phosphospecific ERK antibodies. An ERK2 AEY mutant was generated by the same method.

Cell Culture

Mouse NIH 3T3, rat-kangaroo Ptk$_1$, CHO, human IMR90, MCF7, or HeLa cells were grown in DME supplemented with 10% FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml). Mouse embryonic fibroblasts (MEFs) were provided by Drs. Gille Pages and Jacque Pouyssegur (Centre Antoine Lacassagne, Nice, France) and maintained in the above medium. In some experiments, cells were transfected with cDNA (1 μg) for hemagglutinin (HA)-tagged constitutively active MKK1 or 2 (Mansour et al., 1996), and ERK2 wild-type, inactive, (AEY), or (TEF) mutant using lipofectamine (GIBCO BRL) harvested 16–24 h after transfection.

Cytosol, Membrane, and Nuclear Protein Preparations

Nuclei were separated as described previously (Shapiro et al., 1999). In brief, cells were harvested by scraping into a microfuge tube with extraction buffer containing 10 mM Hepes, pH 7.4, 1.5 mM MgCl$_2$, 10 mM KCl, 1 mM DTT, 0.2 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM PMSF, followed by incubation on ice for 15 min. Passing cells through a 26-gauge needle 10 times was performed to isolate nuclei. The homogenate was centrifuged at 14,000 rpm for 1 min to separate the nuclei from the cytosolic and membrane proteins in the cytoplasmic supernatant. Nuclear proteins in the nuclei pellet were extracted by frequent vortexing in 20 mM Hepes, pH 7.4, 1.5 mM MgCl$_2$, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.2 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM PMSF. The nuclei-free cytoplasmic supernatant and the nuclear protein fraction were further centrifuged for 1.5 h at 100,000 g at 6°C. The supernatant fractions were saved as the cytoplasmic and nucleoplasmic proteins. The pellets containing the cytoplasmic and nuclear membrane proteins were reextracted with SDS-PAGE sample buffer.

Protein Isolation by Sucrose Step Gradient

HeLa cells were transfected with ERK2 wild-type cDNA and allowed to express protein for 16 h on six 10-cm plates. Nocodazole (100 ng/ml) was added for an additional 6 h to three plates. Both untreated and treated cells were rinsed in cold PBS and scraped with 1 ml of 25 mM Hepes, pH 7.4, 1 mM MgCl$_2$, 1 mM DTT, 0.3 M sucrose, 0.2 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM PMSF. The cells were lysed with eight strokes of a dounce homogenizer, and the supernatant was clarified by centrifugation at 3,000 g for 10 min. The supernatant was overlaid onto 1 ml each of 2 M and 1 M sucrose, and centrifuged at 100,000 g for 1.5 h. The 1/2 M sucrose interface was collected and used for immunoblotting.

Immunoblots

For examining pY ERK antibody specificity, cells were directly lysed in SDS-PAGE sample buffer to extract soluble and membrane-bound proteins. Cytosol and membrane proteins (∼40 μg) and the sucrose step gradient fractions prepared as described above were resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane, blocked for 1–2 h with 5% nonfat dry milk in TBS (50 mM Tris, pH 7.5, 0.15 M NaCl, and 0.1% Tween 20), and incubated with primary antibodies diluted in TBS plus 1% BSA for 2 h to overnight. Membranes were washed several times in TBS and incubated with horseradish peroxidase–conjugated anti–mouse or anti–rabbit antibodies (1:10,000; Jackson ImmunoResearch Laboratories). Proteins were detected by enhanced chemiluminescence (NEN Life Science Products/DuPont).

For experiments examining the specificity of the phospho-ERK antibodies, wild-type (His)$_6$-ERK2 containing intact TEY activation site or phosphorylation mutants ERK2 (AEY) and (TEF) were expressed in bacteria, purified by Ni^{+2} NTA chromatography, and phosphorylated by active MKK1 as described (Shapiro et al., 1998). The phospho-ERK immunoblot analysis using SDS-PAGE was performed as described above.

Immunofluorescence

Cells were grown on 18-mm-diameter coverslips (no. 1; VWR) in 6-cm tissue culture plates. After treatment or transfection, coverslips were fixed in 4% paraformaldehyde for 5 min, followed by permeabilization with 0.1% Triton X-100 for 3 min. Alternatively, cells were fixed in cold methanol for 10 min. Coverslips were incubated for 1–2 h in TBS containing 3% BSA, followed by incubation for 1 h at room temperature with the primary antibodies listed above. The coverslips were washed several times with TBS and incubated with FITC- or Texas red–conjugated anti–mouse or anti–rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) and counterstained with DAPI (0.2 μg/ml). Cells were visualized using a Nikon E800 fluorescence microscope, recorded with a Hamamatsu digital CCD camera, and analyzed using IP-LAB Spectrum imaging software.

Golgi structure in transfected cells was classified as described previously (Kano et al., 2000), with modifications. In brief, three phenotypes using the Golgi markers listed above were identified according to whether Golgi structure appeared intact, disrupted, or condensed. Intact Golgi structure was identified by the tubular cisternae in the perinuclear region. The disrupted Golgi structure appeared as a punctate pattern of staining where immunoreactive spots displayed similar length and width dimensions, and were significantly smaller compared with the intact tubular cisternae. The condensed Golgi structure was observed in some transfected cells with reduced cytoplasmic volume. The cells with condensed Golgi structure were not taken into account during the quantitations, as it was difficult to accurately assess the Golgi structure. Under each condition, the number of transfected cells analyzed ranged from 9 to 25, 40 to 90, and 12 to 40 for HeLa, CHO, and MEF cells, respectively.

Results

Association of pY ERK with Golgi Complex

The development of phosphospecific antibodies that recognize mono- and diphosphorylated forms of ERK has proved to be a useful tool for examining ERK regulation (Yao et al., 2000). Using a phosphospecific ERK antibody that recognizes pY ERK within the TEY activation site, we observed a unique staining pattern in asynchronously growing Ptk1 and 3T3 cells (Fig. 1 A). Staining for pY ERK was evident in the nucleus of both cell lines, and in some cells revealed a perinuclear pattern of staining (Fig. 1 A, arrows) that highly resembled the structure of the Golgi complex. Interestingly, it appeared that in the 3T3 cells the perinuclear pY ERK staining was found in a cell that contained condensed chromosomes resembling early prophase of mitosis as evidenced by DAPI staining (Fig. 1 A, bottom), and suggested that the pY ERK staining pattern may be cell cycle regulated.

Because the organization of the Golgi complex is regulated by microtubules, and chemical-induced disruption of microtubules causes fragmentation and dispersal of Golgi stacks into the cytoplasm (Thyberg and Moskalewski, 1999), we compared the localization of pY ERK and the p97 Golgin after nocodazole treatment. Microtubule disruption by nocodazole treatment caused a similar dispersal pattern of p97 Golgin and pY ERK staining to the cytoplasm in both HeLa (Fig. 1 B) and MCF7 (data not shown) cells, further supporting the localization of pY ERK to Golgi vesicles.

To further confirm pY ERK localization to the Golgi vesicles, cells were simultaneously stained for pY ERK and the Golgi protein Mann II. As shown in Fig. 2, the pY ERK only localized the perinuclear region of the cell with condensed chromosomes indicative of mitotic prophase (Fig. 2, second panel from top). The merged image showed a high degree of colocalization of pY ERK and Mann II
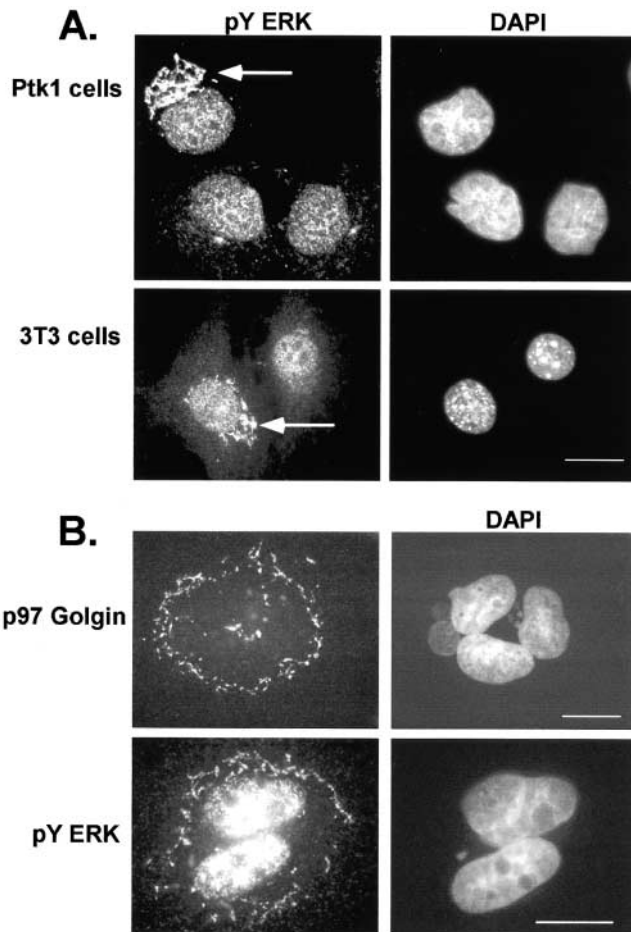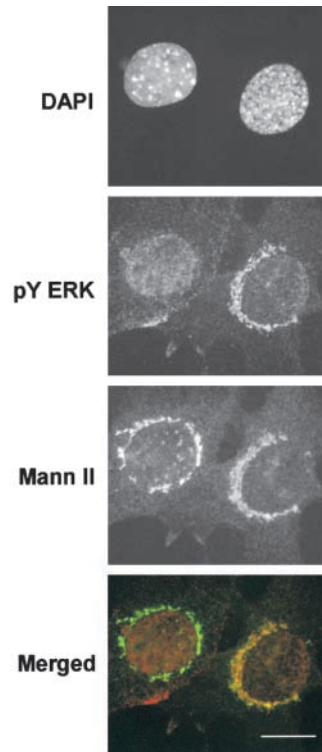
Figure 1. Perinuclear Golgi-like staining of pY ERK2 in cells. (A) Immunofluorescent staining for pY ERK and DAPI in Ptk1 or NIH 3T3 cells. Arrows highlight Golgi-like appearance with pY ERK. (B) Immunofluorescent staining for p97 Golgin and pY ERK in HeLa cells treated with nocodazole for 12 h. DAPI counterstain is shown. Bars, 10 μm.

Figure 2. Colocalization of pY ERK and Mann II in MEFs. MEFs were fixed and costained simultaneously using antibodies against pY ERK and Mann II. (Top) DAPI staining of two cells with the cell on the right side being in prophase of mitosis. The pY ERK and Mann II staining are shown in the second and third panels from the top, respectively. (Bottom) A merged image of pY ERK (red) and Mann II (green). Bar, 10 μm.

(Fig. 2, bottom). In addition, the Mann II staining of the mitotic cell in Fig. 2 appeared more diffuse compared with the nonmitotic cell, and was indicative of early stages of mitotic Golgi fragmentation.

Similar colocalization results were obtained by comparing pY ERK and other Golgi-associated proteins, including Rab6, GM130, and p115 (data not shown). This pattern of pY ERK staining was observed in all mammalian cells tested thus far, including NIH 3T3, HeLa, IMR90, Ptk1, MEF, CHO, and MCF7 cells, as well as with another pY ERK antibody (data not shown). In addition, the pY ERK staining pattern was observed when cells were fixed with paraformaldehyde or cold methanol (data not shown), which further supports the biological relevance of pY ERK found on Golgi complexes and not an introduction of a fixation artifact (Melan and Sluder, 1992). Taken together, these data support the constitutive presence of pY ERK in the nucleus and a cell cycle–specific association with the Golgi complex during mitotic entry.

We have also examined the cellular localization of an antibody raised against pT ERK and found no evidence for localization to the Golgi complex (data not shown). In

contrast, localization of pT ERK was upregulated in prophase cells and found at the region of the kinetochore in mitotic cells (data not shown), and showed a similar staining pattern as described in previous reports using the pTpY ERK antibody (Shapiro et al., 1998).

Determination of pY ERK Antibody Specificity in Cells and In Vitro

We next addressed whether the pY ERK reactivity on the Golgi complex was specific for ERK and not other cross-reactive proteins. For these experiments, we examined the effects of unphosphorylated and tyrosine phosphorylated blocking peptides, which corresponded to the ERK2 peptide sequence used to generate the monoclonal antibody (Yao et al., 2000; see Materials and Methods) on pY ERK staining. Mitotic cells in early prophase were identified after DAPI staining by the presence of condensed chromosomes (Fig. 3, right panels). Incubation of the pY ERK antibody with the pY ERK peptide eliminated most of the nuclear and all of the Golgi staining (Fig. 3, top left). In contrast, untreated cells or incubation with the unphosphorylated ERK peptide had no effect on pY ERK staining in the nucleus or on Golgi complexes (Fig. 3, middle and bottom left). In addition, the ERK peptides had no effect on p97, GM130, or p115 staining on Golgi complexes (data not shown). These data provide strong evidence that the pY ERK staining on the Golgi complex during G2/M is specific for ERK proteins.
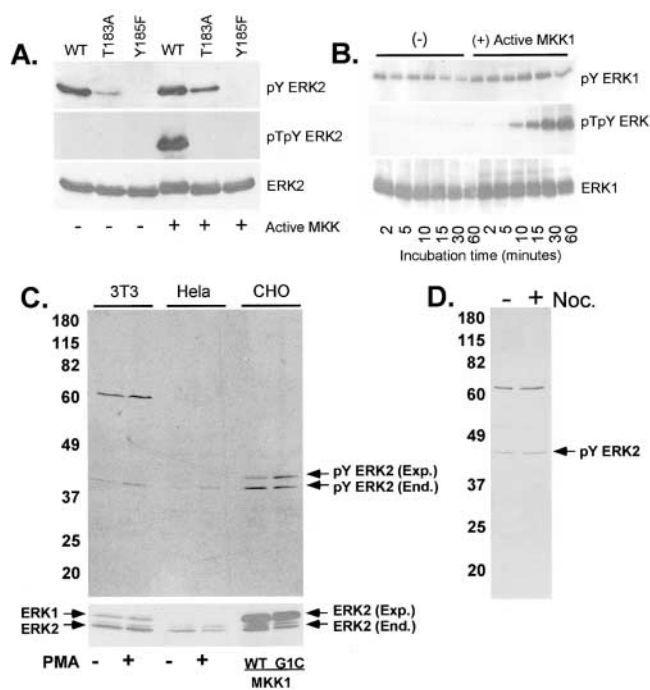
The specificity of the phosphospecific ERK antibodies has been reported previously (Shapiro et al., 1998; Yao et al., 2000). We took additional steps to confirm the pY ERK antibody specificity using ERK2 wild-type (WT) and phosphorylation site mutant-recombinant proteins. In Fig. 4 A (top panel), the pY ERK antibody reacted with wild-
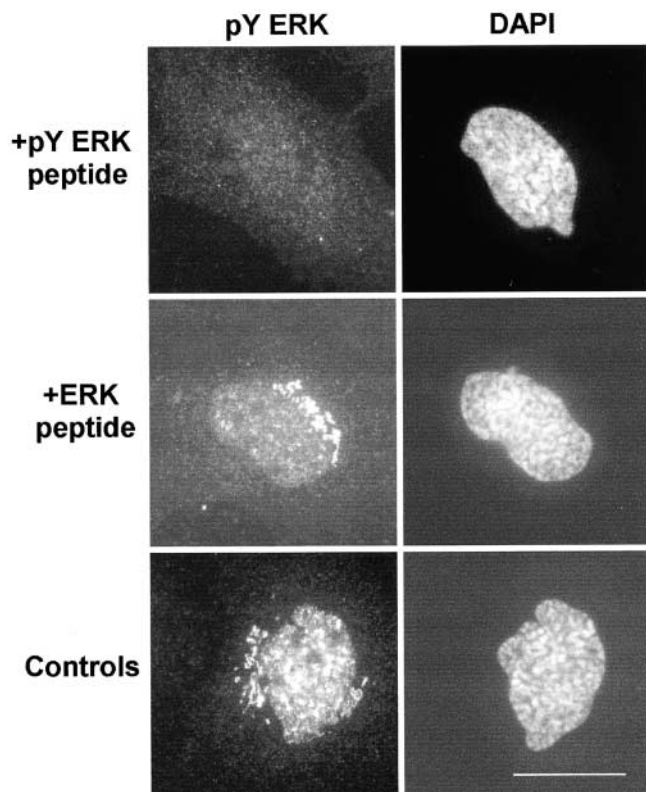
Figure 3. Inhibition of pY ERK staining in mitotic cells with pY ERK–specific peptides. Fixed Ptk1 cells were stained using an antibody against pY ERK that was incubated in the presence of a pY ERK–specific peptide (top), an unphosphorylated ERK–specific peptide (middle), or without peptide (bottom). Using DAPI to identify prophase cells (right), the pY ERK–specific peptide effectively eliminated the nuclear and perinuclear pY ERK staining (left). Bar, 10 μm.

Figure 4. Specificity of the pY ERK antibody in vitro. (A) ERK2 wild-type (WT), and phosphorylation mutants ERK2 T183A and ERK2 Y185F were incubated in the absence or presence of active MKK1. The reaction products were resolved on SDS-PAGE and immunoblotted for pY ERK (top), pTpY ERK (middle), and total ERK2 (bottom). (B) Wild-type ERK1 was incubated in the absence or presence of active MKK1, and the time course of ERK phosphorylation was examined by immunoblotting for pY ERK (top), pTpY ERK (middle), and total ERK1 (bottom). (C) NIH 3T3 or HeLa cells were treated in the absence or presence of phorbol ester (PMA, 10^{-7} M) for 30 min. Proteins were extracted with sample buffer, separated by SDS-PAGE, and immunoblotted for endogenous (End.) or exogenously expressed (Exp.) pY ERK2 (top) or total endogenous and expressed ERK2 (bottom). CHO cells were transfected with wild-type or active (G1C) MKK1 and wild-type ERK2 cDNAs. (D) Immunoblots for pY ERK from lysates harvested as in C from untreated cells and 3T3 cells treated for 6 h with nocodazole (Noc., 100 ng/ml).

type ERK2, to a significantly lesser degree with ERK2 T183A mutant in the absence of active MKK1, and not at all with the ERK2 Y185F mutant. Although addition of active MKK1 caused an increase in pY ERK reactivity towards wild-type ERK2 and ERK2 T183A, no pY ERK reactivity was observed with ERK2 Y185F (Fig. 4 A). It is expected that single amino acid substitution with the ERK2 T183A mutant would decrease the sensitivity to the monoclonal pY ERK antibody, which was generated against 11 amino acids encompassing the TEY activation site (Fig. 4 A, first two lanes). Nevertheless, enhanced pY ERK immunoreactivity in the presence of active MKK1 indicates that the antibody only recognizes pY ERK2. As a control, the antibody against pTpY ERK2 only reacted with wild-type ERK2 in the presence of active MKK1.

The reactivity of pY ERK and pTpY ERK was examined over time in the absence or presence of active MKK. ERK1 in the absence of active MKK1 showed significant pY ERK reactivity, which was slightly enhanced in the presence of active MKK1 (Fig. 4 B, top). In contrast, pTpY ERK reactivity was apparent after incubation for 5 min in the presence of active MKK1 and, as expected, dramatically increased over the course of incubation (Fig. 4 B, middle). Total ERK1 protein is shown for comparison (Fig. 4 B, bottom). In addition, the wild-type ERK pro-

teins in the presence of active MKK1 were the only form of ERK with elevated kinase activity towards myelin basic protein substrate (data not shown), supporting the requirement for both threonine and tyrosine phosphorylation for ERK activation (Seger et al., 1992).

These data indicate that (a) the pY ERK antibody recognizes wild-type ERK and/or pTpY ERK, or (b) the bacterial ERK protein preparations are tyrosine phosphorylated. Several observations support the latter possibility. First, pTpY ERK and pY ERK reactivity superimposes on the slower- migrating phosphorylated form of ERK1 or 2, and not the unphosphorylated ERK which migrates faster on SDS-PAGE. This is not readily apparent in Fig. 4, but the two forms are easily distinguishable on low-percentage bisacrylamide gels. In addition, others have reported that pY ERK is found in the slower-migrating form of ERK, and thus cautioned in using ERK gel shifts as a method for estimating ERK activation (Yao et al., 2000). In our hands, unstimulated ERK bacterial preparations contain some level of the slower-migrating form of ERK1 and 2

that appears to react with the pY ERK antibody (Fig. 4). Other evidence to support the presence of pY ERK in the bacterial ERK preparations is shown in experiments examining pTpY reactivity in the presence of active MKK1 (Fig. 4). The pY ERK reactivity increases only slightly compared with pTpY ERK reactivity with active MKK1, indicating that the tyrosine residue was already phosphorylated. Taken together, the in vitro data indicate that the ERK1 and 2 protein preparations contain phosphorylated tyrosine residues, that the pY ERK antibody is not recognizing unphosphorylated or pTpY ERK, and that phosphorylation of the tyrosine residue within the activation site confers pY ERK antibody specificity.

The specificity of the pY ERK antibody was also tested by immunoblotting of cell protein lysates to determine whether cross-reactivity with other proteins could account for the Golgi staining. As shown in Fig. 4 C (top panel), the pY ERK antibody recognized only endogenous and expressed ERK2 in protein lysates extracted from HeLa, or CHO cells treated in the absence or presence of PMA. In these cells, there was no significant pY ERK immunoreactivity with other size proteins, further supporting this antibody's specificity for recognizing only ERK proteins. Although one cross-reacting protein (∼65 kD) was observed in protein lysates generated from 3T3 cells, it is unlikely that this would be the Golgi protein recognized in the cell staining because it is not observed in the other cell lines. Increased pY ERK1 was also evident with longer autoradiograph exposures, and the MKK inhibitor U0126 blocked pY ERK reactivity in these cells (data not shown). As indicated above, the pY ERK–reactive band superimposed on the gel retarded the slower-migrating form of ERK2 (Fig. 4 C, bottom panel).

The pY ERK antibody was also tested in protein lysates generated from cells treated for 6 h with nocodazole to enrich for cells in G2/M. Under these conditions, ∼30% of the cells were in mitosis as scored by chromosome condensation after DAPI staining, and ∼60% were in G2/M as measured using flow cytometry after propidium iodide staining as compared with ∼10% of untreated cells in mitosis or G2/M (data not shown). Nocodazole-treated cells showed a similar pY ERK pattern of staining as untreated cells, indicating that the pY ERK antibody was not recognizing a nonspecific protein in mitotic cells (Fig. 4 D). We conclude that the Golgi staining observed with the pY ERK antibody is specific for pY ERK, and not due to cross-reactivity with an unrelated Golgi proteins.

### Biochemical Analysis of pY ERK Association with Cell Membranes and Copurification with Golgi Proteins

The subcellular distribution of phosphorylated ERK was examined by immunoblotting after fractionation of cell proteins. NIH 3T3 cells were serum starved for 16 h or treated for 6 h with nocodazole, the cytoplasm and nuclei were separated, and the soluble and membrane protein fractions from the cytoplasm and nuclei were isolated for each treatment. Nocodazole treatment, which significantly enriched the number of cells in G2/M, enhanced the pY ERK reactivity in the cytoplasmic membrane and nucleoplasmic protein fractions (Fig. 5 A, Cyt./M and Nuc./C). In contrast, dually phosphorylated ERK (pTpY ERK) reac-

tivity could be found in all fractions of serum-depleted cells, but showed lower levels in the nuclear membrane fraction of nocodazole-treated cells (Fig. 5 A). The loss of pTpY reactivity under these conditions may be a consequence of enhanced nuclear envelope breakdown. Total ERK2 and MKK1 reactivity was found primarily in both the soluble cytosolic and membrane fractions of the cytoplasm (Fig. 5 A). To validate the quality of the cell fractionations, control immunoblots were performed against proteins whose subcellular localization has been established. As shown in Fig. 5 B, Mann II and nuclear lamin B were found only in the cytoplasmic and nuclear membrane fractions, respectively. As expected, Topo IIα was found enhanced in the nucleoplasm of nocodazole-treated cells (Fig. 5 B), but could be visible in the starved cells with longer autoradiograph exposures (data not shown and Shapiro et al., 1999).

To show additional biochemical evidence that pY ERK associates with Golgi complexes, HeLa cells expressing exogenous wild-type ERK2 were treated in the absence or presence of nocodazole for 6 h to enrich the G2/M population. The Golgi proteins were isolated by sucrose step gradient and the pY ERK reactivity was examined. Nocodazole-treated cells showed a discernible elevation of expressed and endogenous pY ERK compared with the asynchronous untreated cells (Fig. 5 C, upper panel, lane 1 vs. lane 2). Control cells expressing active MKK1 and ERK2 wild-type are shown for comparison (Fig. 5 C, upper panel, lane 3). Importantly, no cross-reacting bands were visible, indicating that the pY ERK antibody was not recognizing a nonspecific protein in asynchronous or G2/M enriched cells (Fig. 5 C). In addition, examination of pTpY ERK in the same samples under identical conditions showed similar reactivity to the positive control compared with pY ERK, but no reactivity with expressed ERK, and a barely reactive band with endogenous ERK (Fig. 5 C). This was not due to a lack of phosphorylated ERK, as nocodazole-treated cell lysates showed a significant elevation of pTpY ERK compared with untreated controls in cell lysates harvested in SDS-PAGE sample buffer as in Fig. 4, C and D (Fig. 5 D). As a control, α-tubulin and p115 expression is shown in the lower panels of Fig. 5 C. Thus, these data support the specific enhancement of pY ERK and association with Golgi complexes during G2/M.

Localization of pY ERK to Golgi Complex Occurs during G2/M Transitions

We confirmed the localization of pY ERK to Golgi complexes during G2/M by staining with an antibody raised against phosphorylated histone H3 (pH3) (Hendzel et al., 1997). Ptk1 cells were used in these studies because of their large size, lower chromosome number, and relatively flat morphology during mitosis. However, similar results have been obtained with HeLa and NIH 3T3 cells (data not shown). The merged images of cells with Golgi-like pY ERK staining (red) were identified and the corresponding pH3 staining (green) confirmed that the observed pY ERK staining correlated with cells that were in G2/M phase of the cell cycle (Fig. 6, left). Three separate examples are shown with the corresponding DAPI staining (Fig. 6, right). The top panels of Fig. 6 are suggestive of a cell in
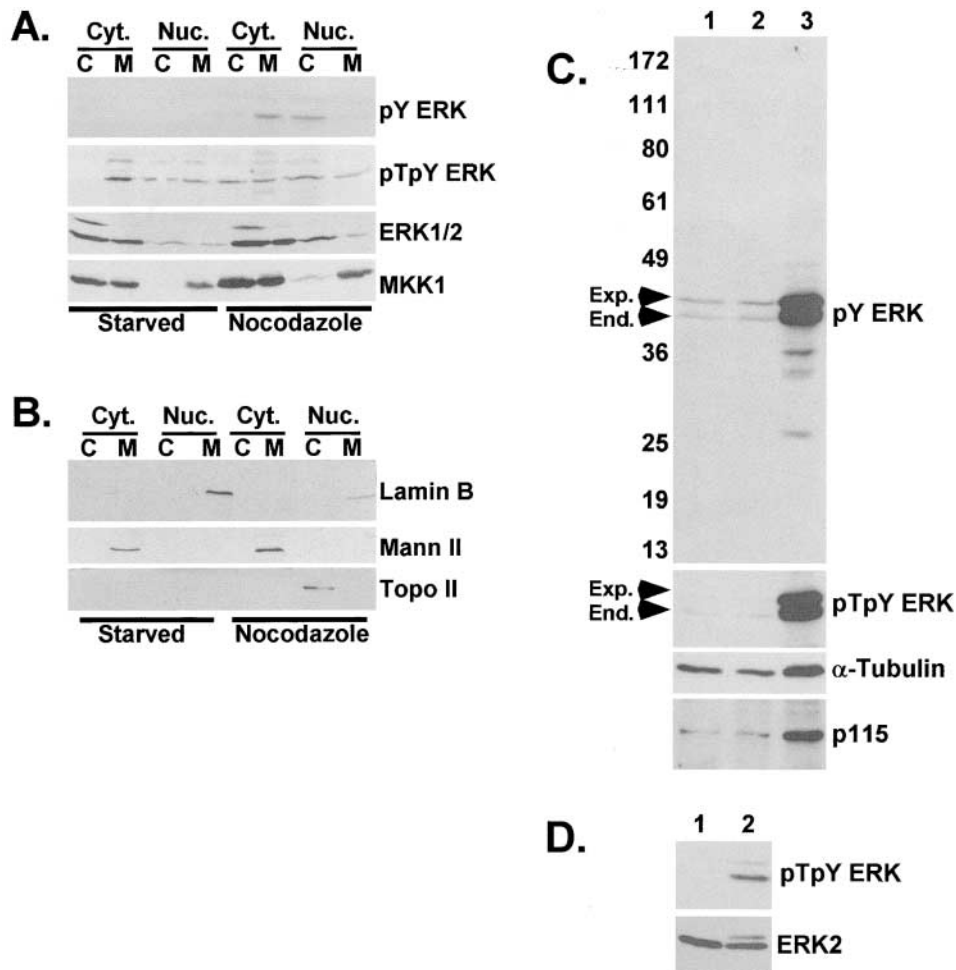
Figure 5. pY ERK associates with cellular membranes of fractionated cells enriched in G2/M. NIH 3T3 cells were incubated in low serum (0.5% FBS) for 16 h (Starved) or treated with nocodazole (100 ng/ml) for 6 h, and the soluble cytosolic (C) or membrane (M) proteins associated with the cytoplasm (Cyt.) or nuclei (Nuc.) were isolated. (A) Immunoblots for pY ERK, pTpY ERK, total ERK2, or MKK1, and (B) for Mann II, lamin B, or Topo IIα from fractionated cell proteins. (C) pY ERK reactivity in proteins isolated by sucrose gradient. Top two panels show immunoblots for pY ERK or pTpY ERK in the 1M/2M sucrose gradient interface from growing (lane 1) or HeLa cells treated with nocodazole for 6 h (lane 2) to enrich for G2/M phase cells. The location of expressed (Exp.) and endogenous (End.) ERK2 protein is indicated with arrows, and the numbers on the left indicate the approximate size in kD. Bottom two panels show immunoblots for α-tubulin and p115 in the same fractions used in the upper panels. Lane 3 contains cell lysates from HeLa cells transfected with active MKK1 and wild-type ERK2 that were harvested by direct lysis in SDS-PAGE sample buffer. (D) Immunoblots for pTpY ERK and total ERK2 from lysates harvested as in Fig. 4, C and D, from untreated and HeLa cells treated for 6 h with nocodazole (100 ng/ml).

late stages of G2 or early prophase where chromosomes have yet to fully condense, the pH3 reactivity is just beginning to be revealed as punctate spots, and the Golgi structure is intact. In contrast, the lower panels clearly show cells that contain progressively condensed chromosomes and high immunoreactivity with pH3. The pY ERK staining in these cells around the area of the Golgi complex is less organized, more dispersed, and indicative of early stages of mitotic Golgi fragmentation.

pY ERK Association with Fragmented Golgi Vesicles throughout Mitosis

Next, we examined pY ERK localization in cells at later stages of mitosis. Fixed cells were double stained by immunofluorescence for pY ERK and Mann II, and mitotic cells were identified using DAPI staining (Fig. 7). The pY ERK pattern of staining in prometaphase cells displayed a punctate appearance similar to that of Mann II and was localized to the spindle poles (Fig. 7, top). Cells in metaphase and anaphase also showed pY ERK staining at the spindle poles, in addition to punctate staining in the region surrounding the poles during metaphase (Fig. 7, middle). This punctate clustering pattern around the spindle poles was also observed with the Golgi-specific p115 and GM130

proteins during metaphase (data not shown). Mann II staining showed punctate staining in the region of the spindle poles and throughout the cytoplasm during metaphase, suggesting some dissociation of pY ERK with Golgi vesicles. Some punctate pY ERK staining around the spindle poles was found in anaphase cells, although this was not as readily evident as during metaphase (Fig. 7, middle). During late telophase, pY ERK is detected on the newly forming Golgi structures in each of the daughter cells (Fig. 7, bottom). These data indicate that pY ERK remains associated with population Golgi vesicles in the region of the mitotic spindles after prometaphase of mitosis, and that it may function in the reformation of Golgi complexes during completion of cell division.

Overexpression of Active MKK1 Alters the Staining Pattern of p115 and Mann II in HeLa and CHO Cells

To establish a direct relationship between the ERK pathway and the Golgi complex, we examined the effects of active MKK1 and ERK phosphorylation on the localization of Golgi proteins. HeLa cells were transfected with cDNAs encoding ERK2 wild-type and MKK1 or 2 wild-type or active MKK1 or 2 mutants. Transfected cells were identified by ERK2 overexpression, and p115 localization
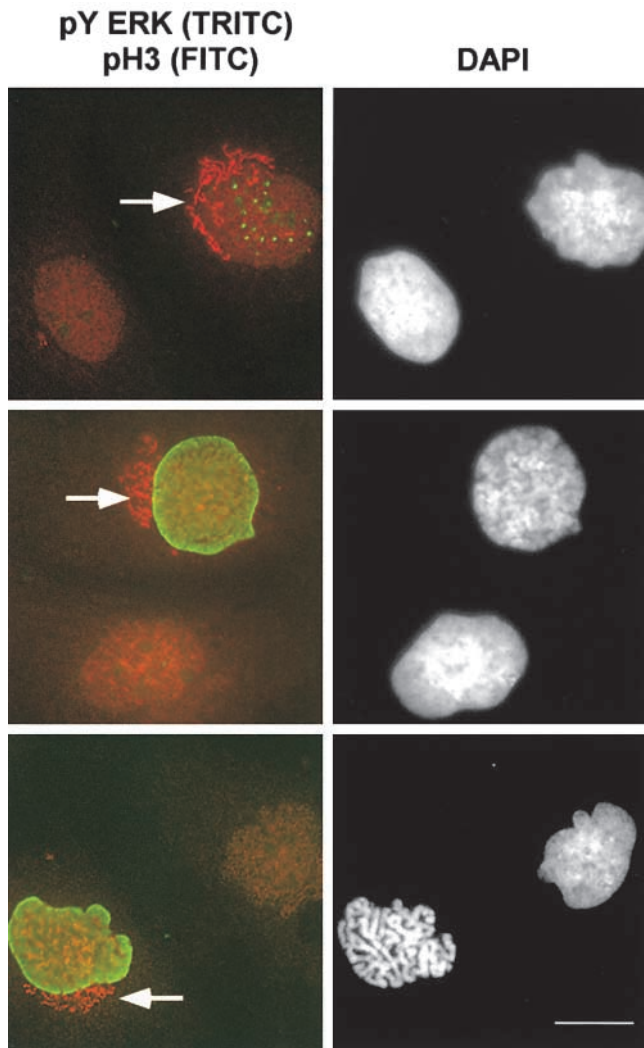
Figure 6. Localization of pY ERK on Golgi complex is specific for cells entering mitosis. G2/M cells were identified by pH3 and DAPI staining in asynchronous Ptk1 cells and the corresponding pY ERK staining was examined. Three separate examples of cells showing progression from late G2 (top) through early (middle) and late prophase of mitosis (bottom) are shown. The arrows in the left panels highlight the perinuclear pY ERK staining in the cells entering mitosis. In all cases where pH3 staining was positive, the cells also showed perinuclear Golgi-like pY ERK staining. Bar, 10 μm.

was monitored simultaneously by immunofluorescence. Three characteristic p115 patterns of staining in the MKK transfected cells were observed corresponding to intact (I), disrupted (D), and condensed (C) Golgi complex (Fig. 8 A, top). Although untransfected cells showed typical intact Golgi structure, the active MKK transfected cells showed a significant number of cells with a diffuse and often punctate p115 staining pattern indicative of disrupted Golgi structure (Fig. 8 A, top left). Furthermore, ~20–25% of the active MKK transfected cells (compared with ~10% of the wild-type transfected cells) showed a reduction in cell volume and a condensed Golgi structure (Fig. 8 A). The staining pattern of p115 and Golgi morphology in the condensed cells could not be accurately evaluated,

thus only intact and disrupted Golgi structures were compared. This was important because many of the smaller cells containing condensed Golgi structure also showed signs of cell death as indicated by the appearance of condensed chromosomes (data not shown), and we wanted to reduce interpretation errors caused by indirect effects of active MKK on Golgi structure.

Confirmation of the presence of active MKK in cells was evident by the increased staining of expressed ERK in the nucleus (Fig. 8 A, middle left panel) compared with cytosolic ERK in the MKK1 wild-type transfected cell (Fig. 8 A, middle right). In addition, the pattern of p115 staining was similar in the MKK1 wild-type transfected cell compared with adjacent nontransfected cells (Fig. 8 A, top right panel). Approximately 50% of the active MKK transfected cells showed a disrupted p115 staining pattern compared with <8% of the wild-type MKK transfected cells (Fig. 8 C, left graph). Similar results were obtained in NIH 3T3 cells (data not shown).

To further examine the disrupted Golgi appearance observed in Fig. 8 A, and to test whether exogenous ERK expression was required, CHO cells were transfected with wild-type or active MKK1, and the Golgi complex was evaluated using Mann II staining. Similar to Fig. 8 A, CHO cells transfected with active MKK show an increase in the number of cells with disrupted Mann II staining and Golgi structure (Fig. 8 B, top panel, transfected cells marked by arrows) compared with untransfected cells. Untransfected and transfected CHO cells were counted and the percentage of cells with disrupted Mann II staining was determined (Fig. 8 C). Expression of wild-type MKK1 enhanced the number of cells with disrupted Mann II staining approximately twofold compared with untransfected cells. This is not surprising, as exogenous expression of wild-type MKK increases ERK phosphorylation and the Golgi complex may be sensitive to these changes. Nevertheless, expression of active MKK1 further enhanced the number of cells with disrupted Mann II staining by twofold compared with wild-type MKK1 transfected cells, supporting a role for MKK and ERK phosphorylation in Golgi structure regulation.

Disruption of Mann II Staining Is Dependent on Tyrosine Phosphorylation of ERK and Not the Kinase Activity of ERK

Next, we addressed whether tyrosine phosphorylation of ERK was required for the disrupted p115 and Mann II staining pattern observed in cells with active MKK, using an ERK2 mutant where the tyrosine within the TEY activation site was mutated to a phenylalanine (TEF). MEF cells were transfected with cDNAs for wild-type or active MKK1 cDNA in combination with ERK2 wild-type (WT), inactive ERK2 (K52R), or ERK2 (TEF) mutant. HA-tagged MKK1 transfected cells were identified as in Fig. 8 B and the Mann II staining pattern was examined. Cells transfected with the active MKK1 showed the disrupted punctate Mann II staining pattern when compared with wild-type MKK1 transfected cells (Fig. 9 A, top). The percentages of intact and disrupted cells were quantified from three separate experiments and cells expressing active MKK1 in the presence of wild-type or inactive ERK show
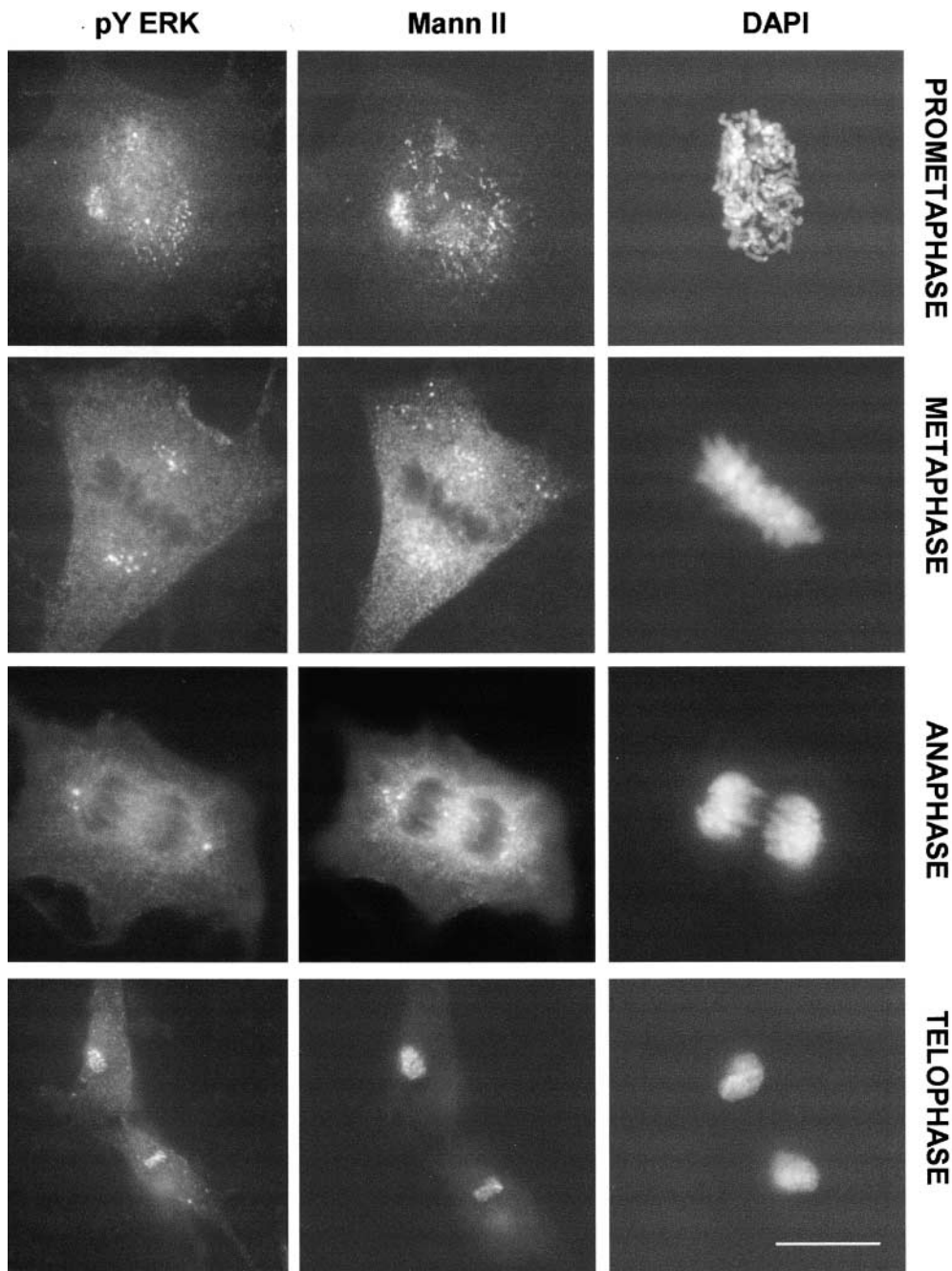
Figure 7. Localization of pY ERK at other stages of mitosis. MEFs were coimmunostained for pY ERK (left) and Mann II (middle). Cells in various stages of mitosis were identified by DAPI staining (right) and the phases of mitosis are shown progressively from prometaphase (top) through telophase (bottom). Bar, 10 μm.

significant increases in the number of disrupted cells compared with wild-type MKK1 transfected cells (Fig. 9 B). Importantly, coexpression of the ERK2 (TEF) mutant reversed the active MKK1-induced disruption of Mann II staining on Golgi complex (Fig. 9 B). Fig. 9 C shows the characteristic Mann II staining pattern of MEF cells transfected with active MKK and wild-type ERK2 and the reversal with expression of ERK2 (TEF) mutant. Similar results were obtained with p115 staining in transfected HeLa cells (data not shown).

We tested for the possibility that pY ERK may regulate Golgi structure indirectly structural changes in the microtubules. Cells transfected with active MKK1 in combination with wild-type ERK2, ERK2 (TEF), or ERK2 (AEY) mutants and allowed to express for 16 h were treated dur-

ing the last 4 h with taxol to stabilize microtubules. Intact or disrupted Golgi structure was evaluated by staining for Mann II as described above. Consistent with Fig. 9 B, increased tyrosine phosphorylation in ERK2 WT or (AEY) expressing cells showed a significant enhancement of the disrupted Golgi phenotype as compared with cells expressing the ERK2 (TEF) mutant. These data strongly support a functional role for pY ERK and not ERK kinase activity in mediating MKK-induced changes in Golgi protein staining patterns.

Discussion

The current data provide the first evidence for the association of an ERK protein, which is partially phosphorylated

A.

B.

C.
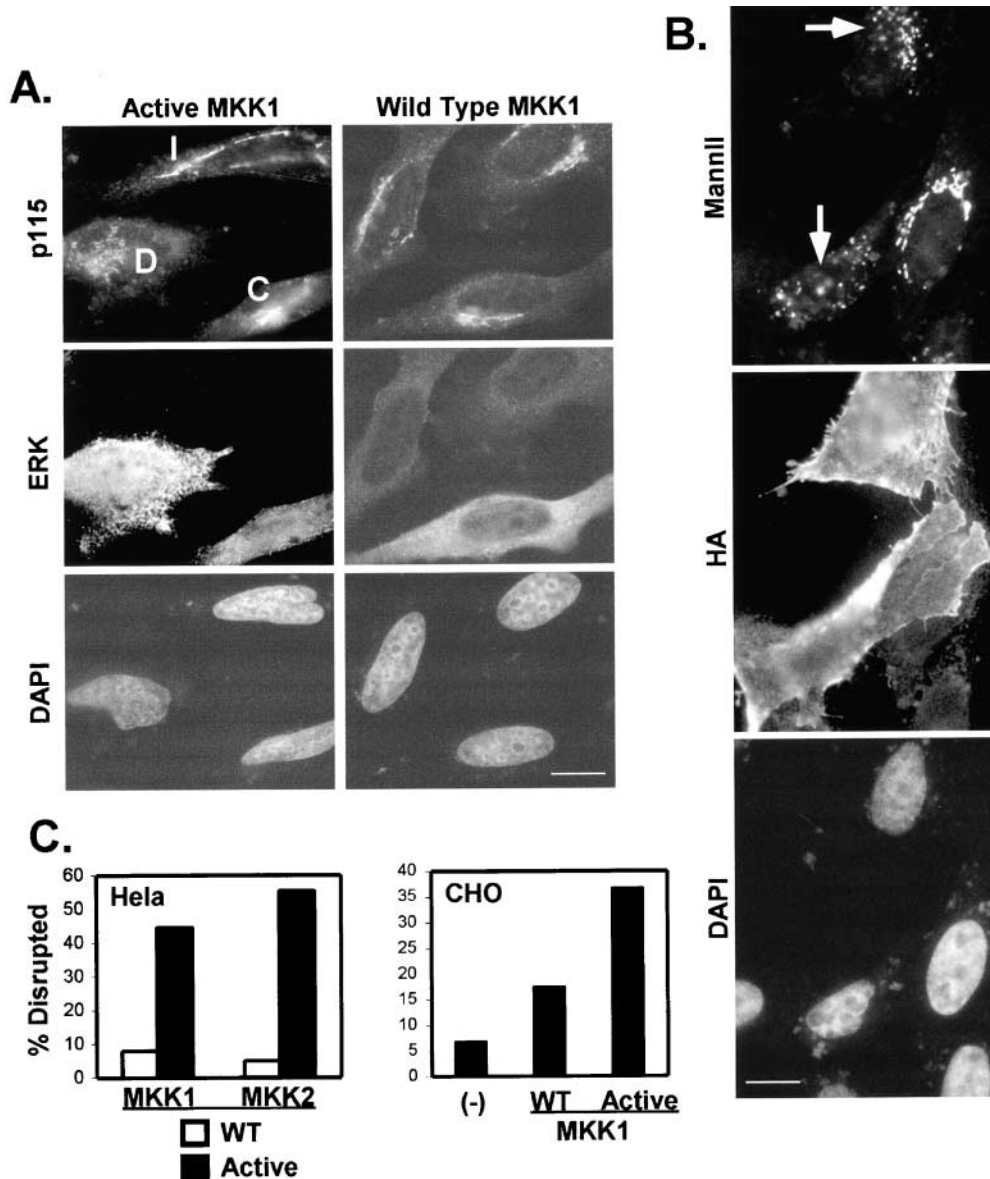
Figure 8. Overexpression of active MKK disrupts localization of Golgi proteins in cells. Intact (I), disrupted (D), or condensed (C) Golgi structure were examined in HeLa (A) or CHO (B) cells transfected with wild-type (WT) or active MKK cDNA in the presence (A) or absence (B) of ERK2 wild-type cDNA. (Top panels) p115 (A) or Mann II (B) in transfected cells. Transfected cells were identified by exogenous ERK in cells expressing wild-type or active MKK1 (A, middle) or by exogenous MKK1 expression (only the condition with active MKK1 is shown in B, middle). (A and B, bottom) DAPI staining. (C) Percentage of disrupted p115 (HeLa) or Mann II (CHO) staining in transfected cells. Data are representative of at least three separate experiments. Bar, 10 μm.

on the tyrosine residue within the TEY activation sequence, with Golgi membrane and vesicles during G2 and M phase of the cell cycle. The pY ERK association with Golgi complex was transient and was found primarily on Golgi complex as cells entered and exited mitosis. This transient association on Golgi complex implies a functional role for pY ERK in regulating mitotic Golgi structure, specifically during fragmentation and reformation. Furthermore, the presence of a single tyrosine phosphorylation within the TEY activation site of ERK suggests that ERK proteins may affect biological functions through mechanisms that are dependent on phosphorylation but independent of kinase activity.

At least three examples report that ERK protein activation is not required for ERK's ability to regulate the function of a transcription factor, phosphatase, or DNA modifying enzyme (Bardwell et al., 1998a; Camps et al., 1998; Shapiro et al., 1999). Similarly, several examples describe a functional activation of the ERK activator proteins MEK1/2 that occurs in the absence of concomitant ERK

activity (Abbott and Holt, 1999; Wright et al., 1999). Thus, pY ERK involvement in the regulation of Golgi complex during mitosis may represent another example in which ERK regulates biological functions through a mechanism that is independent of the ERK kinase activity. Although existing evidence suggests otherwise, it is possible that in vivo, pY ERK has some level of enzymatic activity when associated with Golgi complex. Future studies using synchronized cells expressing ERK phosphorylation mutants may indeed reveal whether pY ERK possesses enzymatic activity during G2/M.

Tyrosine phosphorylation appears to be a key signaling component in the regulation of the Golgi structure. For example, it has been proposed that detyrosinated microtubules are important regulator of Golgi structure whereas tyrosinated microtubules are involved in the structural maintenance of the cell periphery (Thyberg and Moskalewski, 1993). We have shown that enhanced ERK phosphorylation by active MKK expression causes a disruption of the staining pattern of p115 and Mann II Golgi proteins

A.

Active MKK1   MKK1 WT

MannII

DAPI

B.

C.

+ERK2 WT   +ERK2 TEF

D.
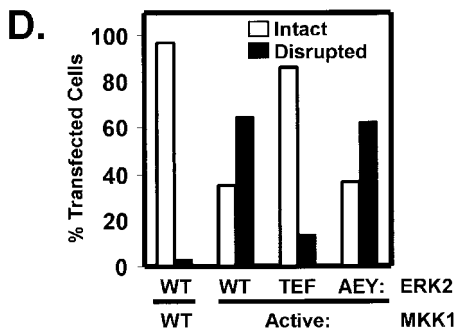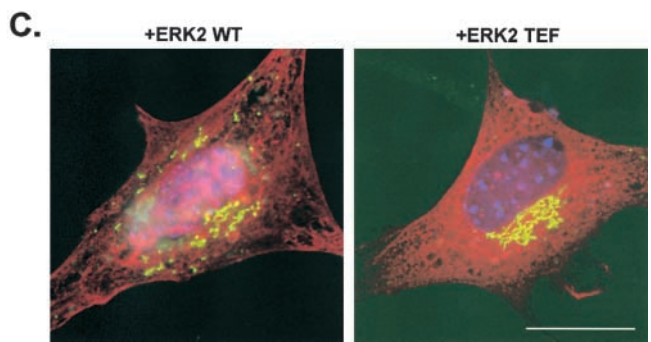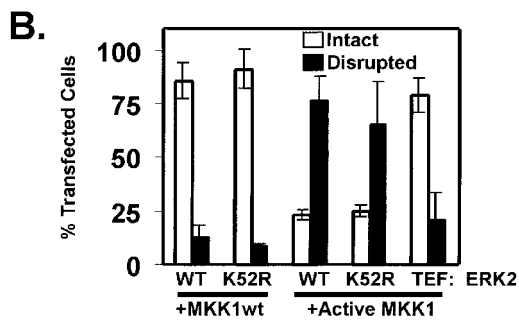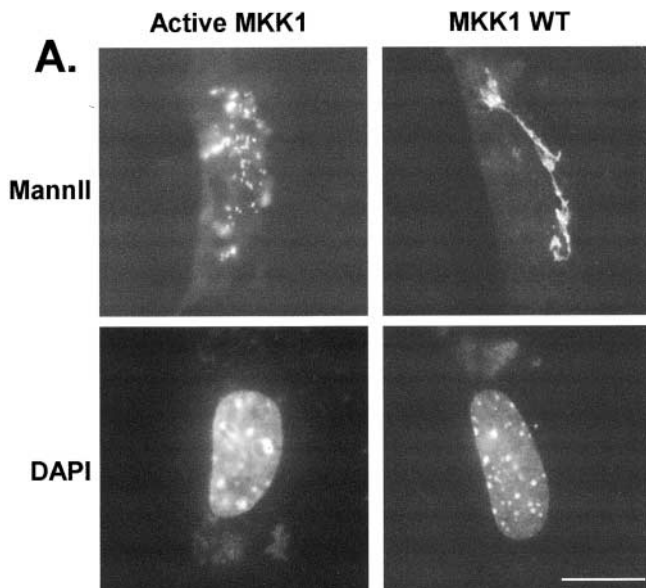
Figure 9. Disruption of Mann II localization requires ERK tyrosine phosphorylation but not ERK activity. (A) MEF cells transfected with active (left) or wild-type (WT) MKK1 (right) plus ERK2 wild-type cDNA were immunostained for Mann II. The punctate Mann II staining pattern in the active MKK1 transfected cell indicates a disruption in the Golgi structure. DAPI staining for each condition is shown for reference. (B) Average percentages of intact (white bars) or disrupted (black bars) Mann II staining in cells transfected with wild-type (wt) or active MKK1 in the presence of wild-type ERK, inactive ERK (ERK2 K52R), or ERK2 tyrosine mutation (ERK2 TEF). The average and SD of each average are shown from three separate experiments. (C) Merged color image of Mann II–disrupted staining in MEF cells transfected with active MKK1 and ERK2 wild-type (WT) (left) or active MKK1 and ERK2 TEF mutant (right). This characteristic staining pattern was used to calculate the values shown in B. HA-transfected cells (red), Mann II (green), and DAPI (blue) are shown in each panel. (D) The percentage of transfected MEF cells with disrupted Golgi complex as scored by Mann II staining was determined after taxol treatment. Cells were transfected and allowed to express wild-type or active MKK1 plus wild-type ERK2, TEF, or AEY mutants for 13 h followed by treatment with taxol (1 μM) for an additional 5 h. Data are representative of two separate experiments. The number of transfected cells examined for wild-type MKK1 plus wild-type ERK2, or active MKK1 plus wild-type ERK2, (TEF), or (AEY) was 33, 42, 51, and 50, respectively. Bars, 10 μm.

(Figs. 8 and 9). Importantly, the disrupted Golgi protein staining could be significantly reversed by coexpression of a nontyrosine phosphorylatable ERK mutant (Fig. 9), indicating that pY ERK is an important component for regulating Golgi structure. Furthermore, an inactive non-ATP hydrolyzing ERK protein, which could still be phosphorylated within the TEY activation site, did not inhibit the ability of active MKK to induce disrupted staining pattern of p115 or Mann II. Evidence that the disrupted staining pattern could also be observed in the presence of taxol to stabilize microtubules (Fig. 9 D) suggests that pY ERK actions on Golgi structure are not through an indirect reorganization of the microtubules. However, definitive proof of this awaits further study, as pY ERK effects on Golgi complex may have occurred before the addition of the taxol. Furthermore, the microtubule organization appeared intact in transfected cells when examining α-tubulin staining (data not shown). Taken together, these data support a role for the pY ERK, but not ERK activity, in

regulating Golgi structure, possibly by regulating protein–protein interactions that promote Golgi complex disassembly or inhibiting protein interactions that maintain intact interphase Golgi structure.

Other cell cycle–regulating kinases may modulate the structure of the Golgi complex. At least two cell cycle–dependent kinases (Cdks) that are regulated by tyrosine phosphorylation, and their interacting cyclins have been found in association with the Golgi complex or involved in mitotic fragmentation. Cdc2/cyclin B is one Cdk that may interact with Golgi stacks in oocytes during mitosis (Leiss et al., 1992) and regulate fragmentation during somatic cell mitosis (Lowe et al., 1998). Another pY kinase, cdk2/cyclin E, has been found in association with the Golgi complex, although its functional significance during the cell cycle has yet to be determined (Gaulin et al., 2000). The serine/threonine polo-like kinase (PlK) is another important mitotic regulator affecting both centrosome maturation and mitotic entry at cyclin B1 degradation and ana-

phase entry (Nigg, 1998). Both PlK and Cdc2 may regulate Golgi structure via interactions with the 65-kD Golgi reassembly stacking protein (GRASP65) (Lin et al., 2000). PlK and Cdc2 can phosphorylate GRASP65 on distinct sites, yet the biological significance of these phosphorylations is not known.

Another function for pY signaling proteins and pY ERK on the Golgi complex may be to regulate protein transport events. Endocytic and exocytic protein trafficking through the Golgi complex is inhibited as cells progress through mitosis (Warren, 1993). One possibility is that pY ERK inhibits protein transport through steric hindrance of Golgi docking proteins, similar to how phosphorylation of GM130 inhibits association with the p115 docking protein during mitosis (Nakamura et al., 1997). In addition, a role for tyrosine kinase and phosphatase activity in the formation of secretory vesicles released from the trans-Golgi network has been suggested previously (Thyberg and Moskalewski, 1993; Austin and Shields, 1996; Gaulin et al., 2000).

The mechanisms responsible for the generation of partially phosphorylated forms of ERK at specific points of the cell cycle are not known. The reported activation of MKK1 proteins during mitotic Golgi fragmentation provides an explanation for the generation of phosphorylated ERK (Colanzi et al., 2000). Conversely, it could be proposed that the balance of serine/threonine and tyrosine phosphatase activities at the Golgi complex is altered during G2/M in a manner that favors increased pY ERK. Protein phosphatase activity is likely involved in regulating Golgi function. A large scaffolding centrosome and Golgi-localized PKN-associated protein (CG-NAP) has been identified that not only interacts with catalytic subunits of protein phosphatase 1 and 2A, but also with protein kinase A (PKA) (Takahashi et al., 1999). Interestingly, a cytosolic protein tyrosine phosphatase, PTP-SL, has been shown to target ERK and is downregulated after phosphorylation on a serine residue by PKA (Blanco-Aparicio et al., 1999). A proposed mechanism for the generation of pY ERK and localization to Golgi complex could involve Golgi-associated PKA targeting and downregulation of protein tyrosine phosphatase. Future experiments will need to address cell cycle–regulated changes in Golgi phosphatase activity and the corresponding ERK phosphorylation. The spatial and temporal regulation of ERK kinases and phosphatases in response to stimuli or cell cycle progression will likely be a determinant of the phosphorylation status and subcellular location of ERK proteins.

In summary, our studies support a role for phosphorylated ERK proteins in regulating the Golgi complex through activity-independent mechanisms. These findings provide an explanation for observed increases in MKK1 activity, in the absence of ERK1 or 2 activities, and involvement in mitotic Golgi fragmentation (Colanzi et al., 2000). Furthermore, our data do not conflict with studies that do not show an involvement of MKK1 in Golgi fragmentation (Lowe et al., 1998; Draviam et al., 2001). In these studies, only active, dually phosphorylated ERK1/2 from the soluble protein fraction (but not the membrane-bound ERK) was examined, and the role of MKK2 was not addressed. In addition, these previous studies reported that a three- to fourfold increase in MKK1 activity above

the endogenous levels was not sufficient to induce Golgi fragmentation in an in vitro system. However, the phosphorylation status of the ERK proteins under these conditions was not reported, and it is not clear whether higher levels of MKK1 activity are needed to affect Golgi structure. The regulation of the Golgi structure and function during mitotic transitions most likely involves a complex array of signaling proteins that regulate structural protein assembly and Golgi protein–protein interactions. Further studies aimed at addressing the specific involvement of ERK proteins in regulating Golgi function may provide new insight into how phosphorylated forms of ERK regulate protein trafficking and protein–protein interactions during mitosis.

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