A Specific Activation of the Mitogen-activated Protein Kinase Kinase 1 (MEK1) Is Required for Golgi Fragmentation during Mitosis

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Abstract. Incubation of permeabilized cells with mitotic extracts results in extensive fragmentation of the pericentriolarly organized stacks of cisternae. The fragmented Golgi membranes are subsequently dispersed from the pericentriolar region. We have shown previously that this process requires the cytosolic protein mitogen-activated protein kinase kinase 1 (MEK1). Extracellular signal–regulated kinase (ERK) 1 and ERK2, the known downstream targets of MEK1, are not required for this fragmentation (Acharya et al., 1998). We now provide evidence that MEK1 is specifically phosphorylated during mitosis. The mitotically phosphorylated MEK1, upon partial proteolysis with trypsin, generates a different peptide population compared with interphase MEK1. MEK1 cleaved with the lethal factor of the anthrax toxin can still be activated by its upstream mitotic kinases, and this form is fully active in the Golgi fragmentation process. We believe that the mitotic phosphorylation induces a change in the conformation of MEK1 and that this form of MEK1 recognizes Golgi membranes as a target compartment. Immunoelectron microscopy analysis reveals that treatment of permeabilized normal rat kidney (NRK) cells with mitotic extracts, treated with or without lethal factor, converts stacks of pericentriolar Golgi membranes into smaller fragments composed predominantly of tubuloreticular elements. These fragments are similar in distribution, morphology, and size to the fragments observed in the prometaphase/metaphase stage of the cell cycle in vivo.

Key words: mitogen-activated protein kinase kinase 1 • extracellular signal–regulated kinases • mitosis • Golgi fragmentation • phosphorylation

Introduction

The pericentriolarly organized Golgi apparatus of mammalian cells undergoes extensive fragmentation during mitosis. This has been known for almost three decades (Robbins and Gonatas, 1964; Lucocq et al., 1987). This fragmentation is observed in mammalian cells and not in plants, yeast, and flies during mitosis (Hirose and Komamine, 1989; Preuss et al., 1992; Stanley et al., 1997). However, the extent of fragmentation, the fate of the fragmented Golgi membranes, the significance of this fragmentation, and above all, the mechanism of the Golgi fragmentation are subject to debate.

To address these questions, we recently reconstituted Golgi fragmentation in permeabilized cells. In this system, normal rat kidney (NRK)[1] cells are permeabilized, washed to remove cytosolic proteins, and then incubated with mitotic extracts prepared from NRK cells arrested in mitosis. The reaction mixture is incubated at 32°C for 60 min, and the organization of the Golgi membranes monitored by fluorescence microscopy using Golgi-specific antibodies. We have shown that mitotic cytosol is highly enriched in cdc2 kinase activity, but that the cdc2 kinase activity is not required for Golgi fragmentation and dispersal of the Golgi fragments from the pericentriolar region. On the other hand, inactivation of the mitogen-activated protein kinase kinase 1 (MEK1) by drugs, or depletion of MEK1 by affinity chromatography or fractionation of mitotic cytosol, results in a loss of Golgi fragmentation. Surprisingly, the cytosolic downstream targets of MEK1, i.e., extracellular signal–regulated kinase (ERK) 1 and ERK2 (MAPKs), are not required for the MEK1-dependent Golgi fragmentation (Acharya et al., 1998).

We now provide evidence that MEK1 undergoes specific activation by phosphorylation during mitosis. The mitosis-specific activation of MEK1 is required for the conversion of the Golgi into tubuloreticular elements, and this fragmentation step does not require cdc2 kinase.

Materials and Methods

Reagents, Antibodies, and Cells

All biochemical reagents were obtained from the sources described before unless otherwise mentioned (Acharya et al., 1998). Nickel agarose beads were purchased from Amersham Pharmacia Biotech. Rabbit anti–rat mannosidase II (MannII) and the anti–NH$_2$-terminal MEK1 antibody were kindly provided by Drs. Kelly Moremen (University of Georgia, Athens, GA) and James Ferrell (Stanford University, Stanford, CA), respectively. The anti–COOH-terminal MEK1 was purchased from Santa Cruz Biotechnology. The phospho-MEK antibody was purchased from New England Biolabs, Inc. Drs. Cesare Montecucco (University of Padua, Padua, Italy) and Nick Duesbery (Van Andel Research Institute, Grand Rapids, MI) kindly provided lethal factor. Recombinant His-MEK1 was a kind gift from Drs. Jocelyn Wright (SUGEN, South San Francisco, CA) and Natalie Ahn (University of Colorado at Boulder, Boulder, CO). Recombinant ΔN7-MEK1 was a gift from Dr. Nick Duesbery. All EM reagents were purchased from the sources described before (Takizawa et al., 1993). Trypsin was purchased from Calbiochem.

All tissue culture cells are grown in complete medium consisting of α-MEM (Gibco) with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO$_2$ incubator.

Preparation of Mitotic and Interphase Extracts

Cytosol from NRK cells arrested in mitosis or interphase was prepared as described before (Acharya et al., 1998). Cell permeabilization and assay for Golgi fragmentation by mitotic extracts were carried out as described before (Acharya et al., 1998).

Incubation of Permeabilized Cells with Lethal Factor–treated Cytosol

Mitotic extract was pretreated with lethal factor (30 ng/ml) for 10 min at 32°C. This mixture and an ATP regeneration system were then added to semi-intact cells and the incubation carried out for 1 h at 32°C. When PD and olomoucine were used, these compounds were added to cytosol pretreated with lethal factor at a final concentration of 75 and 200 μM, respectively. The mixture was incubated for 10 min at 32°C in the presence of an ATP regeneration system and then added to semi-intact cells for 60 min at 30°C to monitor effects on Golgi fragmentation using the permeabilized cell system.

Incubation of Recombinant MEK with Interphase and Mitotic Extract

In a typical preparation, 20 μg of recombinant His-MEK1 (wild-type or ΔN7) was incubated in a total volume of 80 μl with interphase or mitotic extract at a final concentration of 4 mg/ml. The kinase buffer used was composed of 50 mM Hepes, pH 7.2, 10 mM MgCl$_2$, 1 mM DTT, 500 μM ATP, and 30 μCi [^{32}P]ATP. The reaction mixture was incubated for 30 min at 30°C. At the end of the incubation, His-MEK1 was adsorbed of nickel beads, resuspended in 50 mM Hepes, pH 7.2, 200 mM NaCl, 10 mM β-mercaptoethanol, 10 mM imidazole, 10% glycerol, and finally eluted with 200 mM imidazole.

Tryptic Digestion of MEK1

His-MEK1 incubated with mitotic or interphase extract as described above was incubated at room temperature with trypsin (1:100, enzyme/substrate in wt/wt). At the indicated times, aliquots were removed and the reaction terminated by boiling the samples in Laemmli sample buffer. The samples were analyzed by SDS-PAGE followed by autoradiography and immunoblotting with the indicated antibodies as described in the figure legends.

Assay of Enzymatic Activity of MEK1

The procedure was the same as described before (Acharya et al., 1998).

Immunoelectron Microscopy

NRK cells were grown in special petri dishes (Matteck Corporation; Takizawa et al., 1993), permeabilized as described above, and processed for immunoelectron microscopy as described by Polishchuk et al. (1999). For the analysis of intact mitotic cells, NRK cells were incubated for 12 h with 2.5 μg/ml aphidicolin to arrest cells in S phase. The cells were washed by repeated changes of medium and placed in fresh medium. After 6–8 h, the mitotic cells were collected by gentle shake-off and processed for immunoelectron microscopy using anti-ManII antibody followed by HRP-conjugated secondary antibody. Ultrathin sections (80 nm) were observed at 80 KeV with either a JEOL 100C× or 2000F× electron microscope, and semithin sections (0.5 μM) at 400 KeV with a JEOL 4000E× intermediate voltage electron microscope. The mitotic stages were assigned as described by Lucocq et al. (1987).

Results

MEK1 Is Hyperphosphorylated by Mitotic Extracts

It is known that activation of MEK1 is necessary for a number of mitosis-specific events in somatic cells (Acharya et al., 1998; Shapiro et al., 1998; Zecevic et al., 1998). To test whether the mitotic form of MEK1 is in any way different from its activated counterpart found in the nonmitotic stages, wild-type His-tagged MEK1 was expressed in *Escherichia coli* and the recombinant purified protein incubated with mitotic extracts or interphase extracts in the presence of [^{32}P]ATP at 30°C. MEK1 was isolated from the reaction mixtures by adsorption on Ni beads and analyzed by SDS-PAGE and autoradiography (Fig. 1 A). Quantitation of the autoradiogram revealed a fourfold increase in phosphorylation of MEK1 incubated with mitotic vs. interphase extract (Fig. 1 C).
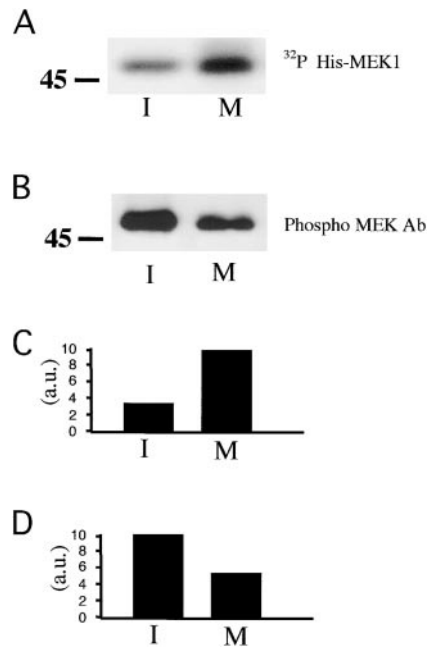
Figure 1. MEK1 is hyperphosphorylated during mitosis and less reactive with the phospho-MEK antibody. His-MEK1 was incubated with mitotic and interphase extracts and [^{32}P]ATP for 30 min at 30°C. The tagged protein was isolated on Ni beads and analyzed by SDS-PAGE followed by autoradiography (A) and subjected to Western blotting with the phospho-MEK1 antibody (B). The quantitation of the autoradiogram and the Western blot is shown in C and D, respectively. His-tagged MEK1 incubated with mitotic extract is fourfold more phosphorylated and twofold less reactive with the phospho-MEK1 antibody compared with incubation in interphase extracts. a.u., arbitrary units.

An antibody that recognizes a generically activated form of MEK1 (phospho-MEK1 antibody) was used to test whether this antibody recognizes the mitotically phosphorylated form of MEK1 compared with MEK incubated with interphase cytosol. The phospho-MEK1 antibody recognizes an activated form of MEK1 phosphorylated at Ser217 and Ser221. This form of MEK1 activation occurs upon the incubation of cells with growth factors or cytokines, or during membrane depolymerization and calcium influx (Crews et al., 1992; Alessi et al., 1994; Robinson and Cobb, 1997). The Ser217- and Ser221-phosphorylated MEK1 activates the downstream kinases called ERKs. An important question is whether the mitotically phosphorylated MEK1 is in some way different from the generically activated form of MEK1. To test this, His-MEK1 was incubated with mitotic cytosol or interphase cytosol and ATP. MEK1 was isolated on Ni beads, released from the beads by imidazole treatment, and analyzed by Western blotting with the phospho-MEK1 antibody. The phospho-MEK1 antibody is twofold less reactive with mitotically activated MEK1 compared with interphase MEK1 (Fig. 1, B and D). Thus, even though MEK1 is hyperphosphorylated during mitosis, it is less reactive with the antibody that recognizes the generically phosphorylated (nonmitotic) form of MEK1.

Mitotically Activated MEK1 Is Differentially Proteolyzed by Trypsin with Respect to Its Interphase Counterpart

His-tagged MEK1 was incubated with either interphase or the mitotic extract in the presence of [^{32}P]ATP for 30 min at 30°C. The tagged protein was isolated on Ni beads and subjected to limited tryptic digestion. At various times the reaction was analyzed by SDS-PAGE and autoradiography and Western blotting with either anti–COOH-terminal or NH$_2$-terminal–specific MEK1 antibody or the phospho-MEK1 antibody. Interestingly, trypsin digestion of mitotically activated MEK, and not interphase MEK1, results in the production of a prominent phosphorylated polypeptide of ~20 kD (Fig. 2 A). This polypeptide is recognized by the COOH-terminal–specific antibody, but not by the phospho-MEK1–specific antibody or the NH$_2$-terminal MEK1 antibody (Fig. 2 B). The mitotic form of MEK1 is less susceptible to digestion with trypsin compared with its interphase counterpart (Fig. 2 B). Thus, the overall susceptibility and pattern of trypsin digestion is different in MEK1 incubated with interphase vs. the mitotic cytosol. The 20-kD polypeptide of MEK1 is not generated when it is incubated with interphase extracts. This would explain the increase in phosphorylation of MEK1 during mitosis, without any increase in reactivity to the phospho-MEK1 antibody. We propose that MEK1, upon phosphorylation by the mitotic extracts, undergoes a change in conformation and this new conformation is differentially sensitive to proteolysis, rendering it active for the Golgi fragmentation process.

Lethal Factor Cleaves MEK1 without Affecting Its Activation by the Upstream Kinase

Bacterial anthrax toxin contains a protein called lethal factor, which cleaves the first eight amino acids from the NH$_2$
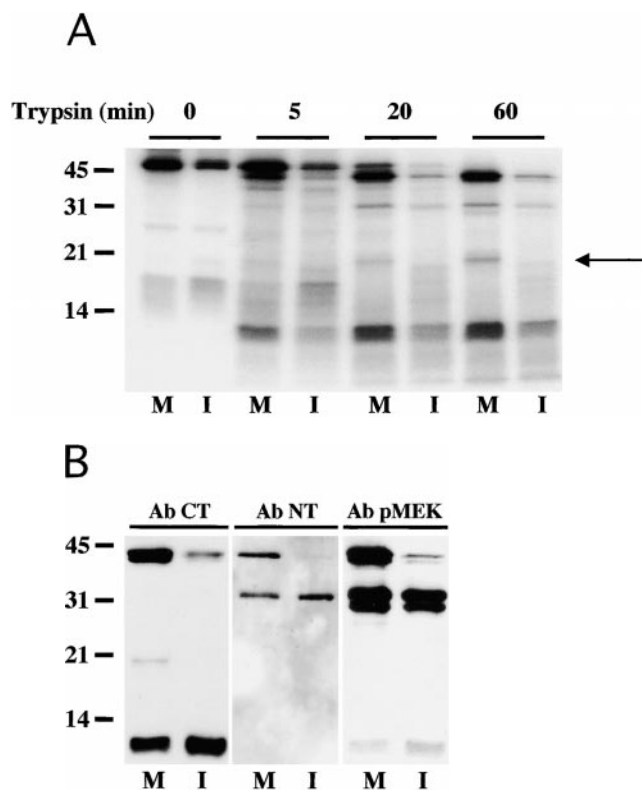
Figure 2. Partial proteolysis of mitotic MEK1 with trypsin generates a specific 20-kD phosphopeptide. His-tagged MEK1 was incubated with mitotic or interphase extract and [^{32}P]ATP for 30 min at 30°C. MEK1 was isolated on Ni beads and released by treatment with imidazole. The phosphorylated His-MEK1 was incubated with trypsin, and at the times shown, analyzed by SDS-PAGE followed by autoradiography (A) and Western blotting with the phospho-MEK1 antibody and antibody against the COOH- and the NH$_2$-terminal of MEK1 (B). Treatment with trypsin results in the generation of a 20-kD phosphorylated species in mitotically activated MEK1 (shown by arrow in A). The 20-kD phosphorylated peptide of MEK1 does not react with the phospho-MEK1 antibody, and reacts with the COOH- and not the NH$_2$-terminal–specific anti-MEK1 antibody. Ab CT, anti-MEK COOH-terminal antibody; Ab NT, anti-MEK NH$_2$-terminal antibody; Ab pMEK, phospho-MEK antibody. M and I are the mitotic and interphase extracts, respectively.

terminus of MEK1 (Duesbery et al., 1998; Vitale et al., 1998). This cleaved form of MEK1 is incapable of binding its downstream target, the MAPKs ERK1 and ERK2. We asked whether the mitotic-specific phosphorylation is possible without the first eight amino acids of MEK1, and whether cleavage with the lethal factor affects MEK1 activity in the Golgi fragmentation process. To address the first question, we used a recombinant form of MEK1 lacking the first seven amino acids. The purified recombinant His-tagged ΔN7-MEK1 was incubated with the mitotic or the interphase cytosol and [^{32}P]ATP for 30 min at 32°C. The ΔN7-MEK1 was isolated on Ni beads and then analyzed by SDS-PAGE followed by autoradiography and Western blotting with the anti–phospho-MEK antibody. Incubation of ΔN7-MEK1 with mitotic extracts results in a fourfold increase in ^{32}P incorporation, and the mutant protein is about fourfold less reactive with the phospho-

A

45 — ^{32}P His-Δn7-MEK1

   I   M

B

45 —   Phospho MEK Ab

   I   M

C

(a.u.)

   I   M

D

(a.u.)

   I   M
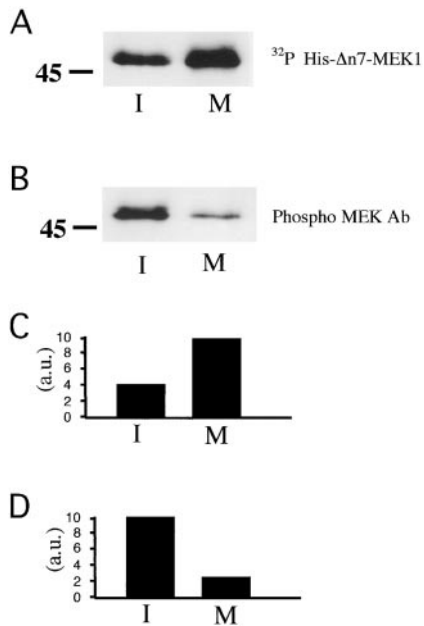
Figure 3. ΔN7-MEK1 is hyperphosphorylated with mitotic extracts. His-tagged ΔN7-MEK1 was incubated with the mitotic and interphase extracts and [^{32}P]ATP for 30 min at 30°C. The tagged version of MEK1 was isolated on Ni beads, released from the beads with imidazole, and analyzed by SDS-PAGE and autoradiography (A) and Western blotting with the phospho-MEK1 antibody (B). The quantitation of the results show that His-ΔN7-MEK is fourfold more phosphorylated when incubated with the mitotic extracts (C) and fourfold less reactive with the phospho-MEK1 antibody (D). a.u., arbitrary units.

MEK1 antibody compared with its counterpart incubated with the interphase cytosol (Fig. 3). Therefore, the wild-type MEK1 and the ΔN7-MEK1 have the same properties with respect to phosphorylation and reaction with the phospho-MEK1 antibody upon incubation with mitotic extract.

Does the treatment of mitotic cytosol with lethal factor affect its Golgi fragmentation activity? To test this, mitotic cytosol was incubated with purified recombinant lethal factor at 32°C for 10 min and the reaction was then divided into three aliquots and used for the following analyses:

(a) The reaction mixture was analyzed by SDS-PAGE followed by Western blotting with the COOH-terminal–specific MEK1 antibody and the NH$_2$-terminal–specific antibody (Fig. 4). Treatment of the cytosol with lethal factor cleaves MEK1. Western blotting with the COOH-terminal–specific antibody reveals a shift in the molecular weight of MEK1 (Fig. 4, top panel). As expected, the NH$_2$-terminal–specific antibody fails to react with MEK1 in cytosol treated with lethal factor (Fig. 4, lower panel), indicating that this treatment is effective in cleaving MEK1.

(b) MEK1 was immunoprecipitated from the lethal factor–treated or -untreated cytosol using the COOH-terminal–specific anti-MEK1 antibody. The immunoprecipitated proteins were incubated with purified recombinant ERK2 for 30 min in the presence of ATP at 30°C. The reaction was then centrifuged to remove MEK1, and the supernatant containing ERK2 was incubated with myelin basic protein (MBP), [^{32}P]ATP at 30°C for 30 min. The


     −   LF

45 —   MEK1 (C)

45 —   MEK1 (N)
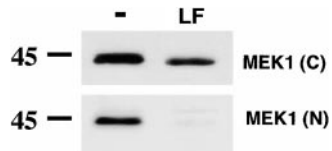
Figure 4. Lethal factor cleaves the first eight amino acids from the NH$_2$ terminus of MEK1. Mitotic extracts were incubated with lethal factor for 10 min at 32°C. The samples were then analyzed by SDS-PAGE and Western blotted with the antibody against the COOH terminus or the NH$_2$ terminus of MEK1. The lethal factor–cleaved MEK1 is increased in mobility and not reactive with the NH$_2$-terminal–specific antibody. −, untreated mitotic extracts; LF, lethal factor–treated mitotic extracts; C, anti–C-terminal MEK antibody; N, NH$_2$-terminal MEK antibody.

reaction was then analyzed by SDS-PAGE and autoradiography and the phosphorylation of MBP taken as a measure of ERK activation. Fig. 5 A shows that cleavage with lethal factor inactivates the ability of MEK1 to activate ERK.

(c) Lethal factor–treated mitotic cytosol was incubated with permeabilized NRK cells that had been prewashed with 1 M KCl. After 60 min of incubation at 32°C, the cells were fixed for immunofluorescence using anti-ManII anti-

A

ERK activation (arbitrary units)

   −   LF

B

% of cells with fragmented Golgi

   −   LF
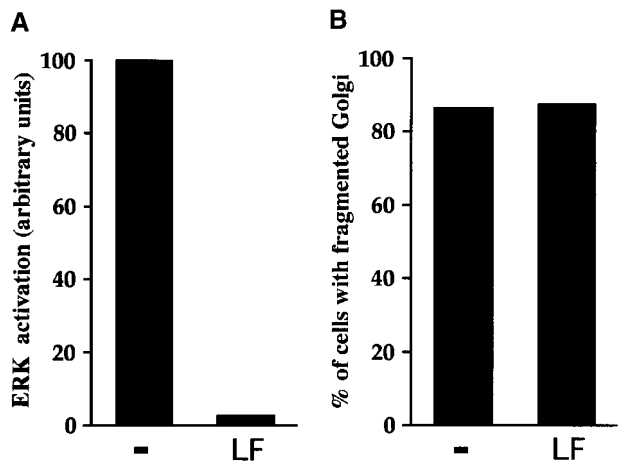
Figure 5. Lethal factor–cleaved MEK1 does not activate ERKs but causes Golgi fragmentation. Mitotic extracts were incubated with lethal factor for 10 min at 32°C. MEK1 was immunoprecipitated with the C-terminal–specific antibody and the complex collected on protein G–Sepharose. The MEK1 on protein G–Sepharose was incubated with ERK2 and ATP for 30 min at 30°C. The sample was centrifuged and the supernatant containing ERK incubated with MBP and [^{32}P]ATP for 30 min at 30°C. The reaction was then analyzed by SDS-PAGE and autoradiography and the level of MBP phosphorylation (a measure of ERK activation) quantitated by PhosphorImager. The results show that lethal factor treatment inactivates the activity of MEK1 towards its substrate ERK2 (A). The aliquot of the cytosol that had been treated with lethal factor (see above) was incubated with permeabilized cells and an ATP regeneration system at 32°C for 60 min. For comparison, an aliquot of cytosol treated with the buffer used for dissolving lethal factor only (−) was similarly tested for effects on the Golgi fragmentation in the permeabilized cell system. The cells were analyzed for the effects on Golgi morphology using anti-ManII antibody by fluorescence microscopy. The results were quantitated by counting 200 cells on two different coverslips and show that lethal factor cleavage does not affect the Golgi fragmentation activity of MEK1 (B).
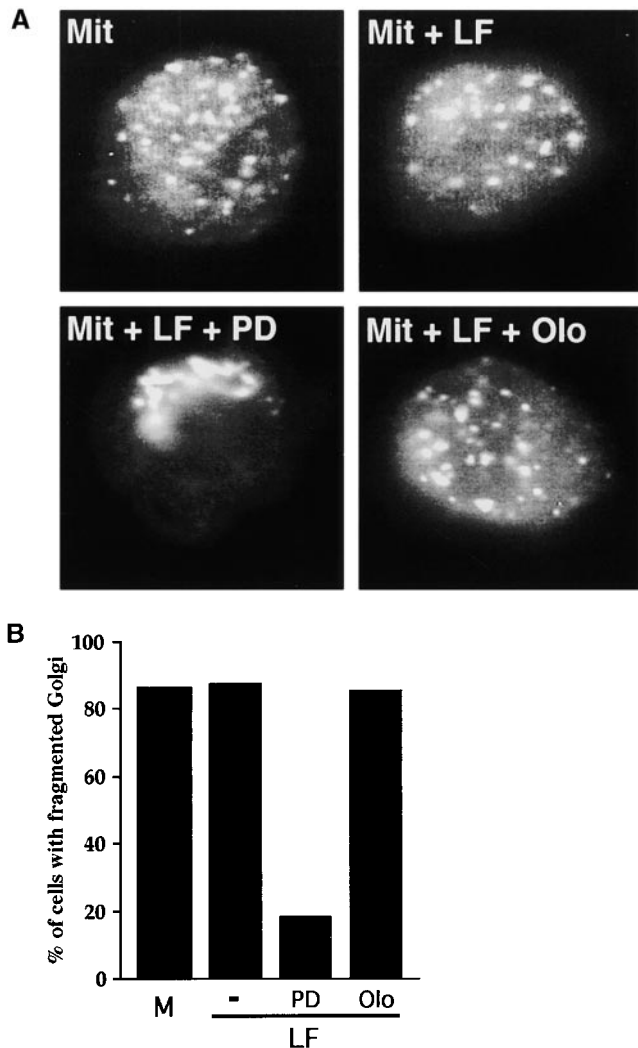
Figure 6. PD and not the cdc2 kinase inhibitor olomoucine inhibit fragmentation of Golgi membranes with mitotic extracts treated with lethal factor in permeabilized cells. Mitotic extracts treated with the lethal factor as described in the legend to Fig. 5 were incubated with either PD or olomoucine and then applied to permeabilized cells. The organization of the Golgi membranes was analyzed by fluorescence microscopy using the anti-ManII antibody (A) and quantitated (B) as described in the legend to Fig. 5. PD and not olomoucine treatment inhibits the fragmentation of mitotic extracts pretreated with lethal factor.

body. 200 cells on two different coverslips were counted, and this quantitation revealed that lethal factor has no effect on the ability of mitotic extracts to fragment Golgi membranes (Fig. 5 B).

### Is cdc2 Kinase Involved in the Fragmentation of Golgi Membranes with Mitotic Extracts Treated with Lethal Factor?

We have shown previously that cdc2 kinase is not required for conversion of the pericentriolar Golgi membranes into smaller fragments (punctate structures at the light microscopy level; Acharya et al., 1998). To confirm that this is

also the case with lethal factor–treated mitotic extracts, they were incubated in the presence of the generic inhibitor of cdc2 kinase activity, olomoucine. This mixture was then applied to permeabilized cells (washed with 1 M KCl) and an ATP regenerating system at 32°C for 60 min. The samples were visualized by fluorescence microscopy. Results show that under conditions where cdc2 kinase is inactive (the cdc2 kinase activity measured in the histone H1 phosphorylation was completely inhibited under these conditions [data not shown]), lethal factor–treated mitotic cytosol can convert the pericentriolarly organized stacks of Golgi cisternae into smaller structures (Fig. 6, A and B).

We have shown previously that an inhibitor of MEK1 activation called PD 98059 (Alessi et al., 1995; Dudley et al., 1995) inhibits Golgi fragmentation (Acharya et al., 1998). Does this compound affect the Golgi fragmentation activity of lethal factor–treated mitotic cytosol? To test this, mitotic cytosol was incubated with lethal factor for 10 min at 32°C and then incubated with PD. The reaction was then supplied to permeabilized NRK cells, prewashed with 1 M KCl and an ATP regenerating system for 60 min at 32°C. The analysis of the cells at the light microscopy level using the anti-ManII antibody reveals a complete inhibition of the Golgi fragmentation (Fig. 6, A and B). Thus, a PD-sensitive activation of MEK1 is necessary for Golgi fragmentation. These results confirm and further support our proposal that the fragmentation of the pericentriolar Golgi stacks during mitosis is by a PD-sensitive, lethal factor–insensitive, activation of MEK1, and does not require active cdc2 kinase.

The Organization of Golgi Membranes in Permeabilized Cells Treated with the Mitotic Extracts

We have shown before that mitotic cytosol causes extensive fragmentation of the pericentriolar Golgi membranes (Acharya et al., 1998). However, the analysis was performed only at the light microscopy level and the organization or the morphology of the fragmented Golgi membranes was not reported because of technical limitations, which we have now resolved. NRK cells were permeabilized, washed with 1 M KCl, and then incubated with mitotic cytosol and an ATP regenerating system. A light microscopic analysis reveals the conversion of the pericentriolar Golgi apparatus (Fig. 7 A) into small punctate structures distributed throughout the cells. A similar preparation was processed for immunoelectron microscopy with an antibody to the cis/medial specific Golgi protein ManII. Thin sections of the cells revealed the presence of ManII in small structures composed of small cisternae and tubuloreticular elements (Fig. 7, B and C–E). Fig. 7 F shows the control Golgi membranes by immunofluorescence and immunoelectron microscopy with anti-ManII antibody in intact nonmitotic NRK cells. A calculation of the size of the Golgi fragments stained with ManII antibody reveals that the stacks of Golgi cisternae of 1.5 μm average size are converted into tubuloreticular fragments of ∼0.60 μm in size by the action of mitotic cytosol. Incubation of the permeabilized cells with cytosol made from interphase cells and an ATP regeneration system at 32°C for 60 min does not affect the pericentriolar organization of the Golgi membranes (data not shown).
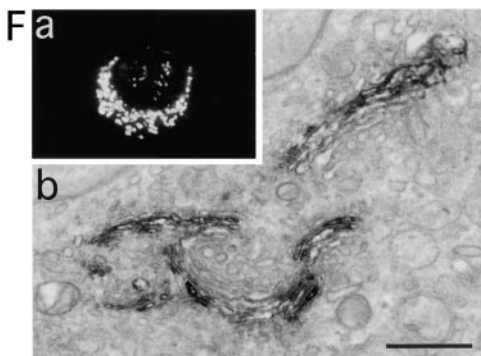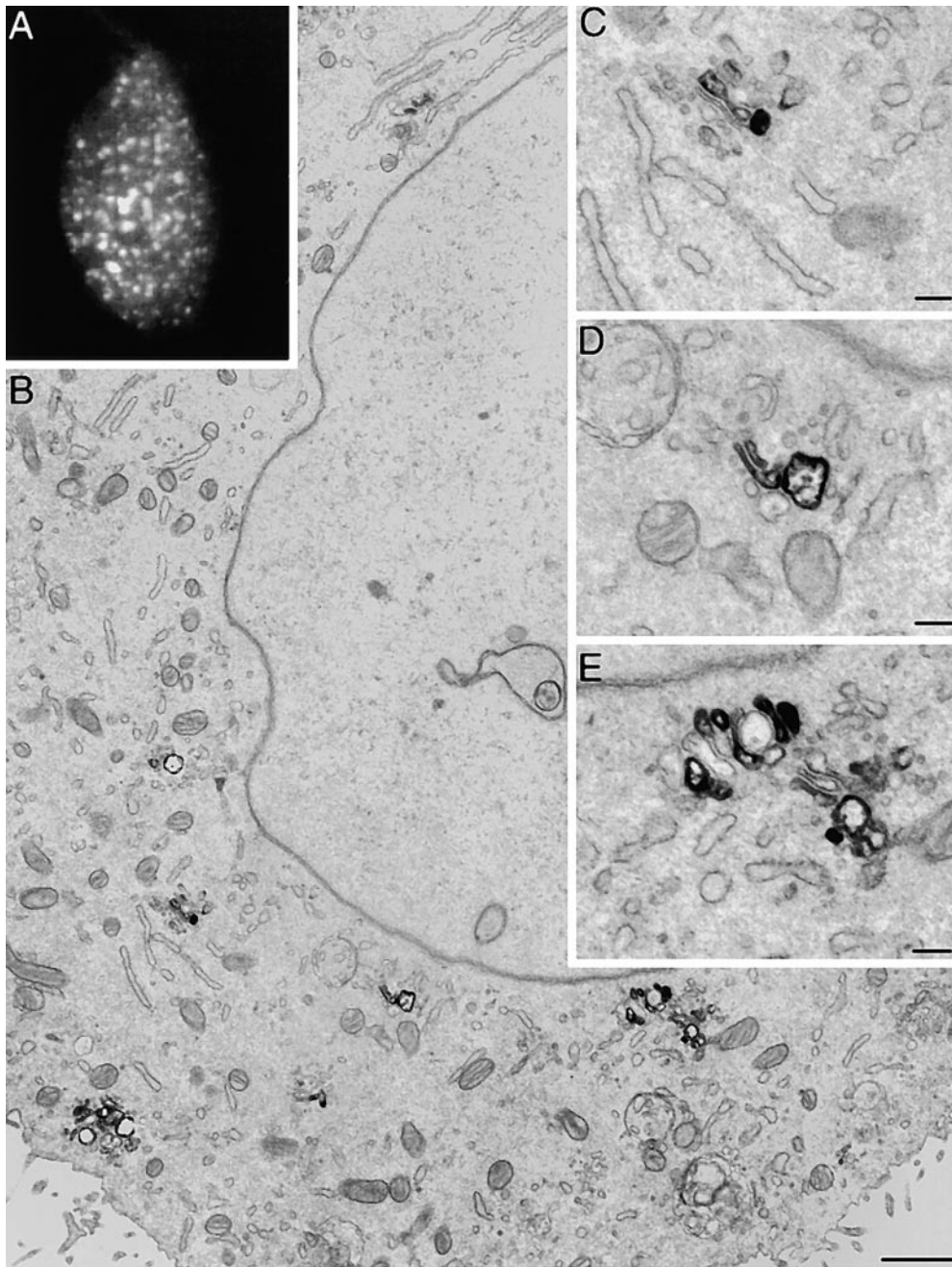
Figure 7. Mitotic extracts convert stacks of pericentriolar Golgi membranes into small tubules and reticular elements in permeabilized cells. NRK cells permeabilized and washed with 1 M KCl were incubated with mitotic extracts and an ATP regenerating system at 32°C for 60 min. The cells were then fixed and analyzed for immunoelectron microscopy using anti-ManII antibody. Thin sections of the cells were visualized and a representative section is shown (B). Treatment with mitotic extracts causes fragmentation and dispersal of Golgi membranes stained with ManII antibody (A, fluorescence microscopic analysis) and the fragments are in the form of small tubules and reticular elements (C–E). (F) a and b are the immunofluorescence and immunoelectron microscopic images of intact interphase cells stained with the Golgi-specific anti-ManII antibody. Bars: 500 nm (B); 200 nm (C–E); and 1 μm (F).

Intact

A

Permeabilized

B

Permeabilized + LF

C
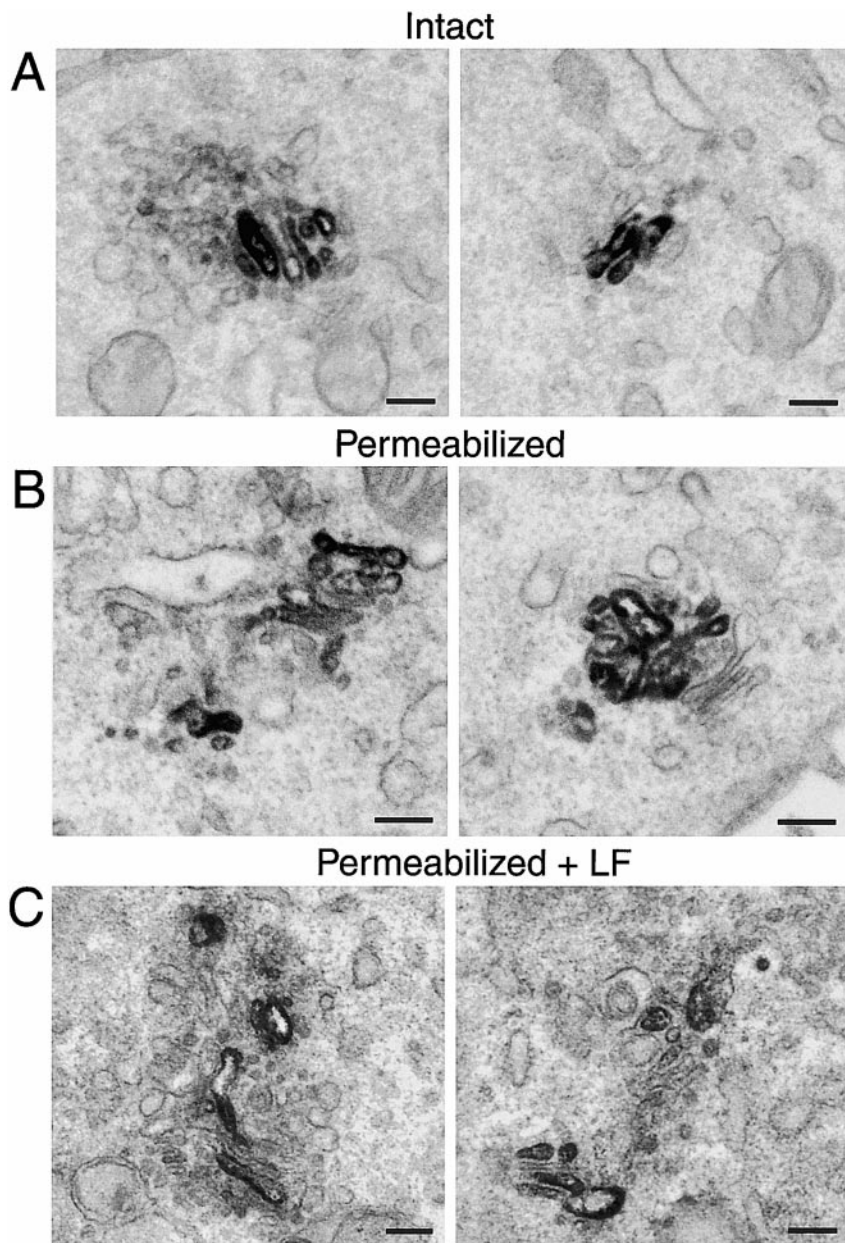
Figure 8. Golgi fragments generated with mitotic extracts treated with or without the lethal factors are the same as those observed in the prometaphase/metaphase mitotic cells in vivo. Intact mitotic cells in the prometaphase/metaphase stage of the cell cycle (A), permeabilized cells incubated with mitotic extracts treated with the lethal factor (C) or untreated (B) were analyzed by immunoelectron microscopy using anti-ManII antibody. Thin sections of the samples were visualized and a representative section of ManII staining Golgi structures is shown. The Golgi fragments are composed of short tubules, reticular elements, and vesicles in all three preparations. Bars, 200 nm.

The Relationship between the Golgi Fragments Generated by Mitotic Cytosol In Vitro and the In Vivo Mitotic Golgi Fragments

Mitotic extracts treated with the lethal factor were incubated with permeabilized NRK cells and an ATP regenerating system at 32°C for 60 min. The samples were fixed and thin sections analyzed by immunoelectron microscopy using anti-ManII antibody. The results of the analysis are shown in Fig. 8 C. Golgi stacks are converted into tubuloreticular membranes and small cisternae in cells incubated with mitotic extract treated with lethal factor. For comparison, we have included the intact mitotic cells (prometaphase/metaphase; Fig. 8 A) and permeabilized cells treated with mitotic extracts (without the lethal factor treatment; Fig. 8 B). The results show that in all cases the Golgi membranes stained with ManII antibody are com-posed of tubuloreticular membranes. The size of these tubuloreticular elements on average is ~0.60 μm. The Golgi fragments in mitotic cells, permeabilized cells incubated with mitotic extracts that are treated with or without the lethal factor, appear morphologically similar and are of similar size and distribution.

Discussion

We have shown that MEK1 is activated specifically during mitosis, and that this activation does not require the presence of the first eight amino acids of MEK. A comparison of the ManII-stained structures in intact mitotic cells in vivo and in our permeabilized cell system shows Golgi membranes in the form of small tubules and reticular membranes. These structures are highly similar in mor-

phology, size (0.60 μm), and distribution (small elements dispersed in the cell), strengthening the proposal that our assay reconstitutes the first step in Golgi fragmentation during mitosis. This process was MEK1-dependent and cdc2 kinase–independent.

Mitosis-specific Activation of MEK1

Our results show that MEK1 is hyperphosphorylated in mitosis. Interestingly, this hyperphosphorylation results in a decreased affinity towards the generic phospho-MEK1 antibody. The mitosis-specific activation of MEK1 is inhibited by pretreatment with PD but not with the lethal factor of anthrax toxin. Thus, the fragmentation of the pericentriolar Golgi stacks into tubuloreticular elements is due to a PD-sensitive and lethal factor–insensitive activity of MEK1, which occurs during mitosis. We believe that this mitosis-specific activation by phosphorylation is responsible for a change in the substrate specificity of MEK1. The 20-kD phosphopeptide of MEK1 is generated only as a result of mitosis-specific phosphorylation. It is also interesting to note that Golgi fragmentation by MEK1 does not require the only known substrates, ERK1 and ERK2. Thus, specific activation by phosphorylation changes the substrate specificity of MEK1 so that it can cause Golgi fragmentation without using the ERKs. How a change in the substrate specificity is achieved based on the phosphorylation state of MEK1 is currently not known.

Warren and colleagues have argued that MEK1 is not required for entry into mitosis and Golgi fragmentation (Lowe et al., 1998). This interpretation is based on experiments in which they loaded cells with the lethal factor and found that this resulted in inactivation of ERK1 and ERK2. The cells entered mitosis and the Golgi membranes were found as clusters of small vesicles (Lowe et al., 1998). However, we can explain their findings based on our results that cleavage with lethal factor prevents activation of ERK1 and ERK2, but this form of MEK is still activated by its upstream kinase and can convert Golgi membranes into tubuloreticular elements (Figs. 5, 7, and 8).

It has also been argued that MEK activity is lower in mitosis and that this is consistent with the inhibition of MEK1 activity by cdc2 kinase. Lowe et al. (1998) suggested that the low MEK1 activity is consistent with MEK1 not being a key mitotic regulator of Golgi fragmentation. We argue that MEK1 activity is low as measured by the activation of ERK1 and ERK2 phosphorylation. However, its activity towards other potential substrates, such as those involved in Golgi fragmentation, spindle dynamics, and checkpoint control may be regulated by an alternative mechanism (Wang et al., 1997; Acharya et al., 1998; Shapiro et al., 1998; Zecevic et al., 1998).

The fact that cdc2 kinase inhibits MEK1 activation is based on in vitro analysis. Whether this also happens in vivo is not known (Rossomondo et al., 1994). Although cdc2 kinase is required for entry into mitosis and controls the fate of many cellular process, this does not mean that all the mitotic-specific events are regulated by cdc2 kinase or that all phosphorylations by cdc2 kinase (in vitro and in vivo) have a physiological significance (Moreno and Nurse, 1990). For example, in the case of lamin disassem-

bly, a recent report shows that this involves sequential phosphorylation first by PKC, which is then followed by cdc2 kinase (Collas, 1999).

Warren and colleagues reported that 50 μM completely inactivated MEK1 in vivo in cells grown in the presence of serum without affecting the ability of cells to enter mitosis or undergo Golgi fragmentation (Lowe et al., 1998). We found that 80 μM PD is required for MEK1 inactivation and inhibits the entry of NRK cells into mitosis (data not shown). We carried out this experiment as follows: NRK cells were treated with aphidicolin (2.5 μg/ml) in complete medium (containing 10% FBS) for 12 h at 37°C. Cells were washed extensively to remove aphidicolin and incubated with fresh medium containing serum. The cells were then washed and incubated with complete medium containing either PD (80 μM) or DMSO (the solvent for PD) at 37°C. After 5 h, the cells were analyzed by fluorescence microscopy with the DNA-specific fluorescent dye HOECHST 33258 to quantitate the mitotic index. In DMSO-treated cells, 30% of the cells were in mitosis, whereas only 5% of the cells were in mitosis in incubations with PD. Our observations are supported by those of Wright et al. (1999), who have recently shown that when PD is applied to cells grown in defined conditions (and not in complete medium containing serum), MEK1 is inhibited and the cells are arrested in G2 (Wright et al., 1999).

What Is the Ultimate Fate of Golgi Membranes during Mitosis?

Our data show that MEK1 is involved in the breakdown of the pericentriolarly organized Golgi membranes into numerous smaller tubuloreticular elements (Acharya et al., 1998; this work). Similar fragments have also been found in cells undergoing mitosis naturally and in cells induced to enter mitosis by okadaic acid treatment (Lucocq, 1992; Lucocq et al., 1995). But do these fragments undergo further processing into clusters of vesicles or fuse with the ER (Thyberg and Moskalewski, 1992; Zaal et al., 1999)? If the Golgi membranes are converted into numerous smaller fragments (resembling tubuloreticular elements), this should be sufficient for their partitioning into the daughter cells. But there is a large volume of data showing that Golgi membranes are converted into clusters of vesicles in mitotic cells (Lucocq et al., 1987; Warren 1993). Jesch and Linstedt (1998) also find that Golgi membranes are converted into vesicles of 60 nm diameter and can be separated from the ER in HeLa cells treated with nocadazole for 24 h. It is possible, however, that these cells are apoptotic not mitotic, and Sesso et al. (1999) have shown that in apoptotic cells, Golgi membranes are found distributed throughout the cell in the form of clusters of 40–60-nm vesicles. Is Golgi vesiculation a property of cells undergoing mitosis, apoptosis, or both?

In summary there is a general agreement in the field that the pericentriolarly organized Golgi apparatus is converted into smaller elements (tubuloreticular elements and smaller stacks) during mitosis. It remains to be determined whether these fragments undergo further processing to form vesicles or fuse with the ER. A comprehensive understanding of Golgi partitioning will require a resolution of this issue, as the process by which Golgi mem-

branes are rebuilt into stacks (postmitosis) will differ depending on the final domicile of the Golgi during mitosis.

References

Acharya, U., A. Mallabiabarrena, J.K. Acharya, and V. Malhotra. 1998. Signaling via mitogen-activated protein kinase kinase (MEK1) is required for Golgi fragmentation during mitosis. *Cell.* 92:183–192.

Alessi, D.R., Y. Saito, D.G. Campbell, P. Cohen, G. Sithanandam, U. Rapp, A. Ashworth, C.J. Marshall, and S. Cowley. 1994. Identification of the sites in MAP kinase kinase-1 phosphorylated by p74Raf-1. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:1610–1619.

Alessi, D.R., A. Cuenda, P. Cohen, D.T. Dudley, and A.R. Saltiel. 1995. PD is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* 270:27489–27494.

Collas, P. 1999. Sequential PKC- and cdc2-mediated phosphorylation events elicit zebrafish nuclear envelope disassembly. *J. Cell Sci.* 112:977–987.

Crews, C.M., A. Alessandrini, and R.L. Erikson. 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. *Science.* 258:478–480.

Dudley, D.T., L. Pang, S.J. Decker, A.J. Bridges, and A.R. Saltiel. 1995. A synthetic inhibitor of the mitogen activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA.* 92:7686–7689.

Duesbery, N.S., C.P. Webb, S. Leppla, V.M. Gordon, K.R. Klimpel, T.D. Copeland, N.G. Ahn, M.K. Oskarsson, K. Fukusawa, K.D. Paull, and G.F. Vande Woude. 1998. Proteolytic inactivation of MAP kinase kinase by anthrax lethal factor. *Science.* 280:734–737.

Hirose, D., and A. Komamine. 1989. Changes in the ultrastructure of Golgi apparatus during the cell cycle in synchronous cultures of catharanthus roseus. *New Phytol.* 111:599–605.

Jesch, S.A., and A.D. Linstedt. 1998. The Golgi and endoplasmic reticulum remain independent during mitosis in HeLa cells. *Mol. Biol. Cell.* 9:623–635.

Lowe, M., C. Rabouille, N. Nakamura, R. Watson, M. Jackman, E. Jamsa, D. Rahman, D.C. Pappin, and G. Warren. 1998. Cdc2 kinase directly phosphorylates the cis-Golgi matrix protein GM130 and is required for Golgi fragmentation in mitosis. *Cell.* 94:783–793.

Lucocq, J.M. 1992. Mimicking mitotic Golgi disassembly using okadaic acid. *J. Cell Sci.* 103:875–880.

Lucocq, J.M., J.G. Pryde, E.G. Berger, and G. Warren. 1987. A mitotic form of the Golgi apparatus in HeLa cells. *J. Cell Biol.* 104:865–874.

Lucocq, J.M., E. Berger, and C. Hug. 1995. The pathway of Golgi cluster formation in okadaic acid treated cells. *J. Struct. Biol.* 115:318–330.

Moreno, S., and P. Nurse. 1990. Substrates for p34cdc2. In vivo veritas? *Cell.* 61:549–552.

Polishchuk, R.S., E.V. Polishchuk, and A.A. Mironov. 1999. Coalescence of Golgi fragments in microtubule-deprived living cells. *Eur. J. Cell Biol.* 78:170–185.

Preuss, D., J. Mulholland, A. Franzusoff, N. Segev, and D. Botstein. 1992. Characterization of the *Saccharomyces* Golgi complex through the cell cycle by immunoelectron microscopy. *Mol. Biol. Cell.* 3:789–803.

Robbins, E., and N.K. Gonatas. 1964. The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* 21:429–463.

Robinson, M.J., and M.H. Cobb. 1997. Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* 9:180–186.

Rossomondo, A.J., P. Dent, T.W. Sturgill, and D.R. Marshak. 1994. Mitogen activated protein kinase kinase 1 (MEK1) is negatively regulated by threonine phosphorylation. *Mol. Cell. Biol.* 14:1594–1602.

Sesso, A., D.T. Fujiwara, M. Jaeger, R. Jaeger, T.C. Li, M.M.T. Monteiro, H. Correa, M.A. Ferreira, R.I. Schumacher, J. Belisario, B. Kachar, and E.J. Chen. 1999. Structural elements common to mitosis and apoptosis. *Tissue Cell.* 31:357–371.

Shapiro, P.S., E. Vaisberg, A.J. Hunt, N.S. Tolwinski, A.M. Whalen, J.R. McIntosh, and N.G. Ahn. 1998. 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. *J. Cell Biol.* 142:1533–1545.

Stanley, H., J. Botas, and V. Malhotra. 1997. The mechanism of Golgi segregation during mitosis is cell type specific. *Proc. Natl. Acad. Sci. USA.* 94:14467–14470.

Takizawa, P.A., J.K. Yucel, B. Veit, D.J. Faulkner, T. Deerinck, G. Soto, M.H. Ellisman, and V. Malhotra. 1993. Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite, ilimaquinone. *Cell.* 73:1079–1090.

Thyberg, J., and S. Moskalewski. 1992. Reorganization of the Golgi complex in association with mitosis: redistribution to the endoplasmic reticulum and effects of brefeldin A. *J. Submicrosc. Cytol. Pathol.* 24:495–508.

Vitale, G., R. Pellizzari, C. Recchi, G. Napolitani, C. Mock, and C. Montecucco. 1998. Anthrax lethal factor cleaves the N-terminus of MAPKKs and indices tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun.* 248:706–711.

Wang, X.M., Y. Zhai, and J.E. Ferrell. 1997. A role for mitogen activated kinase in the spindle assembly checkpoint in XTC cells. *J. Cell Biol.* 137:433–443.

Warren, G. 1993. Membrane partitioning during cell division. *Annu. Rev. Biochem.* 62:323–348.

Wright, J.H., E. Munar, D.R. Jameson, P.R. Andreassen, R.L. Margolis, R. Seger, and E.G. Krebs. 1999. Mitogen-activated protein kinase kinase activity is required for the G(2)/M transition of the cell cycle in mammalian fibroblasts. *Proc. Natl. Acad. Sci. USA.* 28:11335–11340.

Zaal, K.J.M., C.L. Smith, R.S. Polishchuk, N. Altan, N.B. Cole, J. Ellenberg, K. Hirschberg, J.F. Presley, T.H. Roberts, E. Siggia, et al. 1999. Golgi membranes are absorbed into and reemerge from the ER during mitosis. *Cell.* 99:599–601.

Zecevic, M., A.D. Catling, S.T. Eblen, L. Renzi, J.C. Hitte, T.J. Yen, G.J. Gorbsky, and M.J. Weber. 1998. Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. *J. Cell Biol.* 142:1547–1558.