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RAF1-activated MEK1 is found on the Golgi apparatus in late prophase and is required for Golgi complex fragmentation in mitosis

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A mitotically activated mitogen-activated protein kinase 1 (MEK1) fragments the pericentriolar Golgi stacks in mammalian cells. We show that activated MEK1 is found on the Golgi apparatus in late prophase. The fragmented and dispersed Golgi membranes in prometaphase and later stages of mitosis do not contain activated MEK1. MEK1-dependent Golgi complex fragmentation is through activation by RAF1 and not MEK1 kinase 1. We propose that a RAF1-dependent activation of MEK1 and its presence on the Golgi apparatus in late prophase is required for Golgi complex fragmentation.

Introduction

Mammalian cells contain ~100 stacks of Golgi cisternae organized in a contiguous ribbonlike structure concentrated in the pericentriolar region. In mitosis, the Golgi membranes are highly fragmented and dispersed throughout the cell. This fragmentation is thought to be necessary for partitioning Golgi membranes between daughter cells during cell division (Warren, 1993). Evidence in support of this proposal comes from the recent findings that preventing fragmentation and dispersal of the pericentriolar Golgi apparatus prevents mammalian cells from entering mitosis (Sutterlin et al., 2002).

We have taken the following approach to identify components involved in the fragmentation of pericentriolar Golgi apparatus. Normal rat kidney (NRK)* cells grown on coverslips are permeabilized with ice-cold digitonin and washed with a buffer containing 1 M KCl to remove cytosolic and loosely attached peripheral membrane proteins. The permeabilized and salt-washed cells are incubated at 32°C for 60 min with cytosol prepared from mitotic NRK cells. The organization of Golgi membranes is monitored by fluorescence microscopy using antibodies to Golgi complex–specific proteins such as mannosidase II (ManII). As a result of this procedure, the Golgi apparatus is fragmented and dispersed in tubulo-reticular membranes throughout the cytoplasm (Acharya et al., 1998; Colanzi et al., 2000). We have previously shown that mitotically activated mitogen-activated protein kinase 1 (MEK1) and Polo-like kinase 1 are required for Golgi complex fragmentation (Acharya et al., 1998; Colanzi et al., 2000; Sutterlin et al., 2001). We now report that RAF1 is required to activate MEK1 for Golgi complex fragmentation during mitosis and that mitotically activated MEK1 is found on the Golgi membranes in late prophase.

Results and discussion

RAF1-dependent activation of MEK1 is necessary for Golgi complex fragmentation

There are at least two different kinases known to activate MEK1: RAF1 and MEK kinase 1 (MEKK1; Garrington and Johnson, 1999). RAF1 regulates cell proliferation in response to growth factors, cytokines, and hormones (Morrison and Cutler, 1997). It is not clear how RAF1 activity is regulated, but a Ras-dependent membrane recruitment, phosphorylation on multiple sites, and binding to several proteins (such as 14–3-3 and heat shock proteins 90/50) are some of the key factors (Kolch, 2000). The activation of RAF1 during mitosis follows a different and specific mechanism that does not require Ras or binding to membranes, but involves the protein kinase Pak1 (Ziogas et al., 1998; Laird et al., 1999; Zang et al., 2001, 2002).

We tested whether RAF1 is required for MEK1-dependent mitosis-specific Golgi complex fragmentation. There are no known RAF1-specific inhibitors and it is also difficult to

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*Abbreviations used in this paper: ERK, extracellular signal-regulated kinase; ManII, mannosidase II; MEK1, mitogen-activated protein kinase 1; MEKK1, MEK kinase 1; NRK, normal rat kidney; ppMEK, phospho-MEK; RKIP, RAF1 kinase inhibitory protein.

Key words: mitogen-activated protein kinase kinase 1; cell cycle; organelle inheritance; RKIP; phosphorylation

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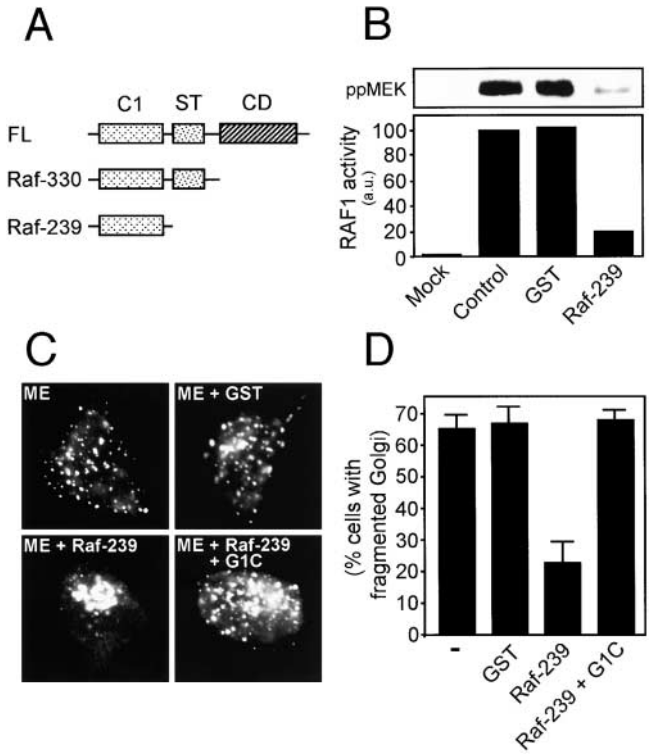

Figure 1. **Raf-239 inhibits endogenous RAF1 activation and Golgi complex fragmentation.** (A) A schematic diagram of RAF1 domains. Full-length (FL) RAF1 is composed of a COOH-terminal catalytic domain (CD) and NH_2-terminal regulatory domain containing a cysteine-rich domain (C1) and a serine/threonine rich-domain (ST). (B) Mitotic cytosol (350 μg) was incubated with buffer alone, 50 μg GST, or 15 μg Raf-239 for 10 min at 32°C. Endogenous RAF1 was immunoprecipitated with 2 μg anti-RAF1 (C-12) antibody complexed to beads. The samples containing immunoprecipitated RAF1 were incubated with 3 μg recombinant MEK1 for 10 min at 32°C in the presence of ATP. At the end of the incubation, MEK1 was recovered and immunoblotted with anti-ppMEK antibody. The quantitation of the results is shown in the lower panel. The extent of MEK1 phosphorylation is used as an indication of RAF1 activity. Raf-239 inhibits activation of the endogenous RAF1 by 75%. The quantification of the experiment shown in the figure is presented; similar results have been obtained in four different experiments. (C) Mitotic extract treated with Raf-239 as described above was applied to permeabilized cells. The organization of the Golgi membranes was analyzed by fluorescence microscopy using the anti-ManII antibody. (D) Quantitation of the effects of Raf-239 on Golgi complex fragmentation by mitotic cytosol. Raf-239 inhibited the mitotic-specific Golgi complex fragmentation. The addition of 3 μg of bacterially expressed constitutive active MEK1 (G1C) restored Golgi complex fragmentation. The data represent the average of seven different experiments.

deplete RAF1 from mitotic cytosol without using detergents. Therefore, we used the following strategy to specifically block RAF1 activation. We expressed a GST-tagged deletion mutant of RAF1 (corresponding to amino acids 1–239) in *Escherichia coli* (Fig. 1 A). This peptide corresponds to the autoinhibitory domain of RAF1 and acts as dominant negative inhibitor of RAF1 when expressed in mammalian cells (Flory et al., 1998). Purified recombinant GST-RAF1/1-239 (Raf-239) was added to mitotic cytosol for 10 min at 32°C with ATP. The endogenous RAF1 was immunoprecipitated from this incubation mixture using an antibody against the COOH terminus of RAF1. The immunoprecipitated RAF1 (on beads) was incubated with recombinant MEK1 and ATP at 32°C. The sample was centrifuged to separate MEK1 (supernatant) from RAF1 (beads). RAF1 is known to phosphorylate MEK1 at serines 218 and 222 and the extent of this double phosphorylation is a reliable indicator of RAF1 activity (Bondzi et al., 2000). The supernatant containing MEK1 was analyzed by SDS-PAGE followed by Western blotting with an antibody that recognizes MEK1 phosphorylated at serines 218 and 222 (anti–phospho-MEK [ppMEK] antibody). Incubation of mitotic cytosol with recombinant Raf-239 inhibited RAF1 activation (Fig. 1 B). Quantitation of the Western blot revealed that Raf-239 causes a 75% inhibition of RAF1 activity toward MEK1 (Fig. 1 B, bottom). Similar results were obtained with GST-RAF1/1-330 (Raf-330), which corresponds to the entire regulatory domain of RAF1 (Fig. 1 A). For the experiments described below, we used Raf-239 because it was easier to express and purify. Raf-239 most likely interferes with the complex network of proteins that regulates RAF1 function titrating out activating components (Bruder et al., 1992; Flory et al., 1998). Raf-239 does not titrate out MEK1 because the MEK1 binding sites are located in the COOH-terminal catalytic domain of RAF1 (Yeung et al., 2000).

We then tested the effect of Raf-239 on the Golgi complex fragmentation induced by mitotic cytosol in permeabilized cells. Mitotic cytosol was preincubated for 10 min at 32°C in the presence of ATP and Raf-239 and added to permeabilized NRK cells for 60 min. Cells were then fixed and analyzed by immunofluorescence microscopy. Addition of 15 μg of Raf-239 to the assay inhibited Golgi complex fragmentation in >70% of the cells (Fig. 1, C and D). Importantly, addition of recombinant constitutively activated MEK1 (G1C) alleviated the inhibition of Golgi complex fragmentation induced by Raf-239. Thus, inhibition of Golgi complex fragmentation by Raf-239 is a consequence of specific inhibition of RAF1-dependent MEK1 activation.

An additional strategy was used to interfere with the RAF1–MEK1 pathway by using RAF1 kinase inhibitory protein (RKIP). In the presence of RKIP, RAF1 cannot bind MEK1 (Yeung et al., 2000). RKIP does not inhibit MEKK1-mediated phosphorylation and activation of MEK1 (Yeung et al., 1999). RAF1 and MEKK1 were immunoisolated from mitotic cytosol using anti-RAF1 and anti-MEKK1 antibodies conjugated to Sepharose beads, respectively. The beads containing immunoisolated RAF1 and MEKK1 were incubated with MEK1, RKIP, and ATP at 32°C for 10 min. The reaction mixture was centrifuged to separate beads from soluble MEK1. The supernatant containing MEK1 was analyzed by SDS-PAGE and Western blotted with anti-ppMEK antibody. Our results show that RKIP inhibited RAF1-mediated phosphorylation of MEK1, but did not affect MEKK1-mediated phosphorylation of MEK1 (Fig. 2 A). This is in agreement with the work of Yeung and colleagues (Yeung et al., 1999). Recombinant purified RKIP was incubated with mitotic cytosol and this sample added to the assay reconstituting Golgi complex fragmentation process. RKIP strongly inhibited

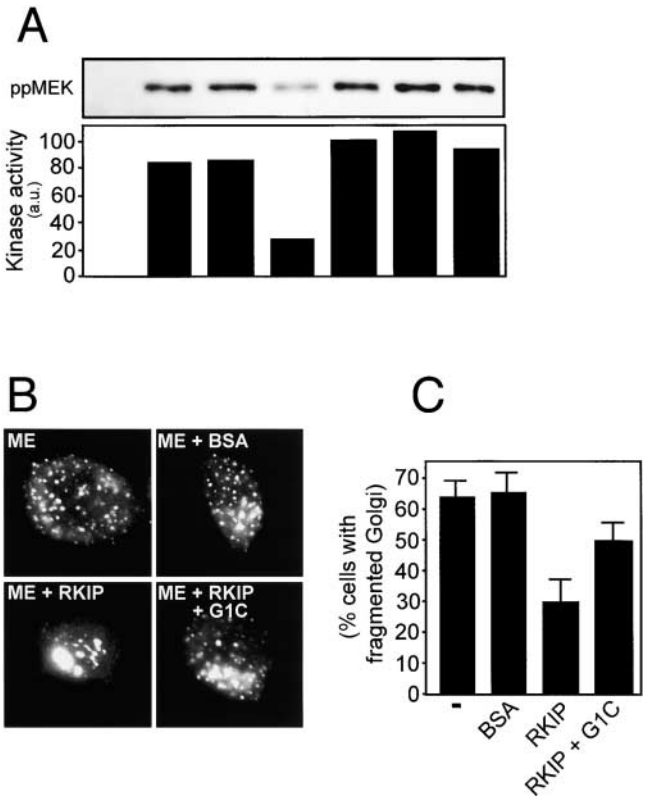

Figure 2. **RKIP does not block MEKK1-dependent MEK1 activation, but inhibits RAF1-dependent MEK1 activation and Golgi complex fragmentation.** (A) RAF1 and MEKK1 were immunoprecipitated from 250 μg mitotic cytosol using 2 μg of C-12 and 43-Y antibodies, respectively. The beads were subsequently incubated with 3 μg recombinant MEK1 for 10 min at 32°C in the presence of buffer alone, 50 μg BSA, or 15 μg RKIP. MEK1 was recovered by centrifugation and immunoblotted with anti-ppMEK antibody. Quantitation of the results is shown in the lower panel. RKIP inhibits RAF1-dependent MEK1 activation. The quantification of the experiment shown in the figure is presented; similar results have been obtained in three different experiments. (B) Mitotic extract treated with RKIP as described above was added to permeabilized cells. The organization of the Golgi membranes was analyzed by fluorescence microscopy using the anti-ManII antibody. (C) Quantification of the effects of RKIP on Golgi complex fragmentation by mitotic cytosol. Inhibition of the RAF1-dependent MEK1 activation inhibited the mitotic-specific Golgi complex fragmentation. The addition of 3 μg of bacterially expressed constitutive active MEK1 (G1C) restored the Golgi complex fragmentation. The data represent the average of six different experiments.

mitotic cytosol–dependent Golgi complex fragmentation (Fig. 2, B and C). Addition of recombinant constitutively activated MEK1 (G1C) significantly restored Golgi complex fragmentation activity of mitotic cytosol containing RKIP. The restoration of Golgi complex fragmentation activity was partial because RKIP is required in molar excess concentration to efficiently inhibit RAF1-dependent MEK1 activation (Yeung et al., 1999). The addition of higher concentration of G1C, sufficient to fully revert the inhibition, is not feasible in our assay because it dilutes the concentration of mitotic cytosol necessary for Golgi complex fragmentation. These results provide strong evidence that RAF1-mediated activation of MEK1 is required for Golgi complex fragmentation by mitotic cytosol. We have previously shown that mitotically activated MEK1 is conformationally different from its functional counterpart in interphase cells (Colanzi et al., 2000). Partial proteolysis of His-MEK1 incubated with mitotic cytosol reveals a 20-kD phosphopeptide, which is not observed upon proteolysis of His-MEK1 incubated with interphase cytosol. RAF1 was isolated from mitotic cytosol and incubated with His-MEK1. This preparation of His-MEK1 was subjected to partial proteolysis. The resulting proteolytic fragments did not contain the 20-kD phosphopeptide described above (unpublished data). Together, these results suggest that RAF1 is required, but is not sufficient for complete mitosis-specific activation of MEK1. The identity of additional components required for MEK1 activation in Golgi complex fragmentation is not known.

Inhibiting RAF1 activation prevents entry into mitosis

It has recently been suggested that RAF1 is specifically activated during mitosis (Laird et al., 1999; Zang et al., 2001, 2002). However, formal proof that RAF1 is required to enter mitosis is lacking. NRK cells were arrested in S-phase with aphidicolin. The cells were subsequently washed to remove aphidicolin, 5 h after aphidicolin removal, 400 cells were injected either with Raf-239 or GST. 2 h after injection, the cells were fixed and visualized by fluorescence microscopy to monitor the organization of DNA and Golgi apparatus. The mitotic index of the injected cells was determined relative to that of the noninjected cells in the same coverslip. The mitotic index of cells injected with GST was 91 ± 11. The mitotic index of cells injected with Raf-239 was 41 ± 8 (Fig. 3). Thus, injection of Raf-239 inhibited entry into mitosis in >50% of cells. These results strengthen the proposal that a RAF1–MEK1-mediated process is necessary for entry into mitosis in mammalian cells.

Activated MEK1 is found on the Golgi membranes during late prophase

Our previous findings revealed that MEK1-mediated Golgi complex fragmentation was independent of its well-known cytosolic substrates called extracellular signal-regulated kinases (ERKs; Acharya et al., 1998; Colanzi et al., 2000). This suggested that MEK1 could directly bind to Golgi

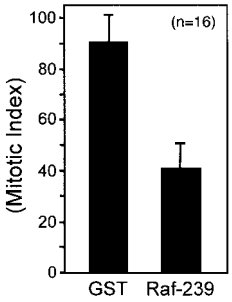

Figure 3. **RAF1 is required for entry into mitosis.** NRK cells were arrested in S-phase with aphidicolin. The cells were washed to remove aphidicolin and injected 5 h later with Raf-239 or GST. After an additional incubation for 2 h, the cells were stained with Golgi complex and DNA-specific markers. 400 cells were counted for each experimental condition to calculate the mitotic index. The mitotic index is the ratio of the percentage of mitotic cells in the pool of injected of cells compared with noninjected cells on the same coverslip. The mitotic index of cells injected with GST was found to be 91 ± 11 as compared with 41 ± 8 for the Raf-239–injected cells. The data are calculated from 16 independent experiments.

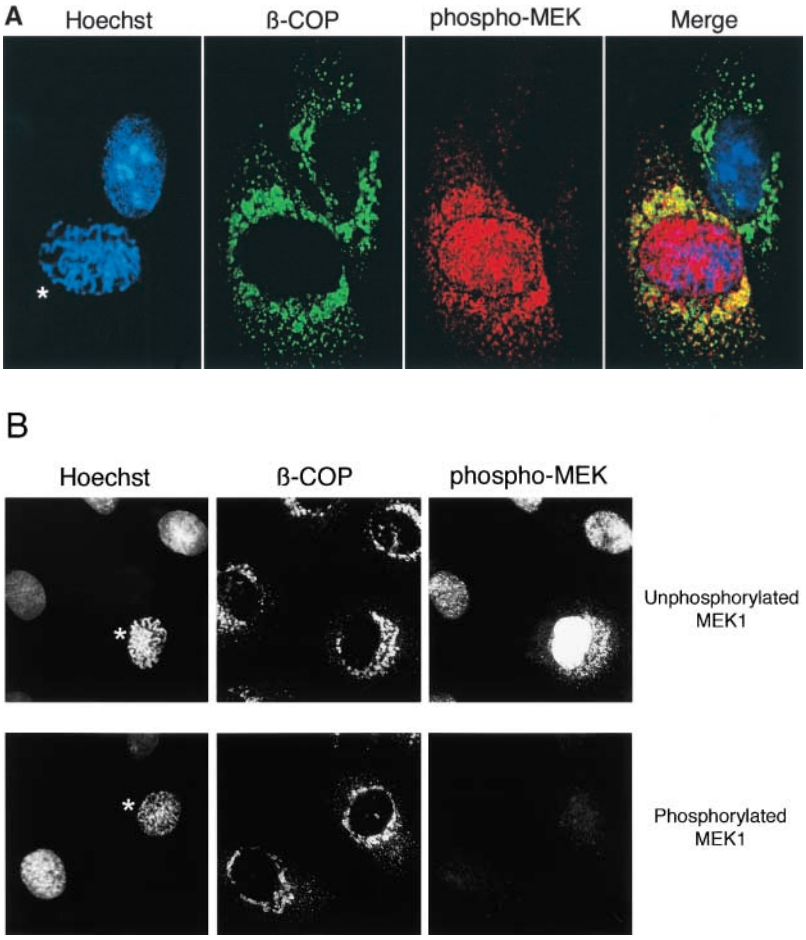

Figure 4. **Activated MEK1 is found on the Golgi apparatus in late prophase.** NRK cells were blocked in S-phase with aphidicolin. The cells were washed to remove aphidicolin. 7 h after aphidicolin removal, cells were processed for fluorescence microscopy to visualize the Golgi apparatus, activated MEK1, and DNA with antibodies against β-COP, ppMEK, and the DNA-specific dye Hoechst, respectively. (A) Confocal microscopy image showing that activated-MEK1 is localized to the Golgi apparatus in late prophase (condensed DNA*) and not in interphase (uncondensed DNA). (B) Epifluorescence microscopy image showing cells stained with ppMEK antibody that had been preincubated with either mitotically activated MEK (phosphorylated MEK1) or unphosphorylated MEK1. Preincubation of ppMEK antibody with mitotically phosphorylated MEK1 abolished the Golgi complex–specific staining of cells in late prophase (condensed DNA*). Therefore, ppMEK antibody staining is due to the presence of activated MEK1 on the Golgi apparatus.

membranes and initiate fragmentation via a Golgi complex–specific substrate. If this is correct, MEK1 should bind to Golgi membranes during the fragmentation process. The cellular localization of activated MEK1 during mitosis was examined by staining NRK cells with anti-ppMEK antibody (Willard and Crouch, 2001). The cells were also stained with Hoechst, and antibodies to the Golgi protein β-COP or Giantin to identify the mitotic stage and reveal the organization of Golgi membranes, respectively.

Activated MEK1 showed nuclear localization during prophase (Fig. 4 A). This is in agreement with the fact that MEK1 translocates to the nucleus during this mitotic stage (Tolwinski et al., 1999). Interestingly, activated MEK1 was localized to the Golgi apparatus in late prophase (Fig. 4 A). To ensure that the Golgi complex–specific staining of ppMEK antibody was due to interaction with activated MEK1, cells were stained with ppMEK antibody that had been preincubated with either mitotically activated recombinant MEK1 (phosphorylated MEK1) or unphosphorylated recombinant MEK1. Preincubation of ppMEK antibody with mitotically phosphorylated MEK1 abolished the Golgi complex and nuclear staining. ppMEK antibody staining on the Golgi apparatus in late prophase, therefore, is due to its binding to the mitotically activated MEK1 (Fig. 4 B). The percentage of the β-COP positive structures that contained mitotically activated MEK1 was 47 ± 6%.

Table I. **Quantitation of cells containing activated MEK1 on Golgi membranes during mitosis**

Stage	Fraction of cells containing phospho-MEK on Golgi membranes	Cells containing phospho-MEK on Golgi membranes
		%
Interphase	0/50	0
Early prophase	0/35	0
Middle prophase	2/46	4
Late prophase	24/29	83
Prometaphase	4/50	7
Metaphase to cytokinesis	0/50	0

NRK cells treated as described in Fig. 4 were labeled for activated MEK1 and DNA and imaged by fluorescence microscopy. The mitotic stages were defined based on the level of chromosome condensation. The number of cells with activated MEK1 on the Golgi complex (numerator) and the number of cells counted (denominator) are shown. 83% of cells in late prophase contain activated MEK1 on Golgi membranes.

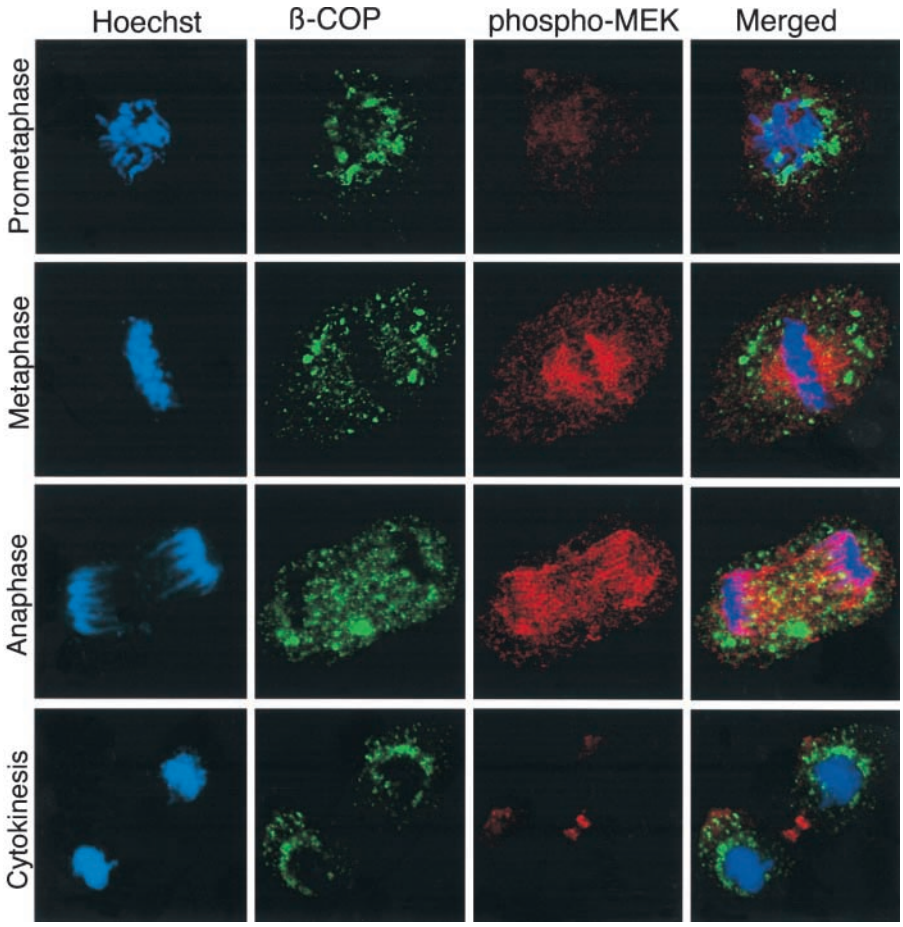

Figure 5. **Intracellular distribution of activated MEK1 during mitosis.** Cells were treated as described in Fig. 4 A and processed for confocal fluorescence microscopy by staining the Golgi apparatus, activated MEK1, and DNA with antibodies against β-COP, ppMEK, and the DNA-specific dye Hoechst, respectively.

Similar results were obtained in HeLa, NIH, and COS-7 cells (not depicted).

The number of cells with Golgi complex localized activated MEK1 was quantified and the results are shown in Table I. About 83% of cells in late prophase exhibited Golgi complex–specific localization of mitotically activated MEK1. Cells at mitotic stages before or after late prophase did not contain mitotically activated MEK1 on Golgi membranes (Fig. 4 A and Fig. 5). Together, these finding provide strong evidence that mitotically activated MEK1 is present on the Golgi membranes in late prophase.

Activated MEK1 is found attached to astral microtubules in metaphase, polar microtubules in anaphase, and finally in the central spindle microtubules in cells in cytokinesis (Fig. 5). Therefore, activated MEK1 interacts with different cellular components to (most likely) catalyze different reactions depending on its location.

Conclusions

The timing of the appearance of activated MEK1 on the Golgi apparatus coincides with the initiation of Golgi complex fragmentation during mitosis. One of the components necessary for the activation of MEK1 in the Golgi complex fragmentation process is RAF1. Circumstantial evidence indicates that an ERK-like protein may be the downstream target of MEK1. An ERK-like protein or a mitotically activated ERK with novel modification has been found on the Golgi membranes (Acharya et al., 1998; Cha and Shapiro, 2001). It is also reported that ERK2 phosphorylates the Golgi complex–associated complex protein GRASP55 (Jesch et al., 2001). It is conceivable that a RAF1–MEK1–ERK/ERK-like pathway induces the phosphorylation of GRASP55 during mitosis and consequently destabilizes the Golgi stacks. This reaction, along with reactions catalyzed by Polo-like kinase 1 and the cdc2 kinase, may well explain the mechanism of Golgi complex fragmentation during mitosis (Lowe et al., 1998; Sutterlin et al., 2001).

Materials and methods

Reagents, antibodies, and cells

All biochemical reagents were obtained from the sources described before unless otherwise mentioned (Acharya et al., 1998; Colanzi et al., 2000). The anti–COOH-terminal MEK1 (C-18), RAF1 (C-12), and MEKK-1 (43-Y) were purchased from Santa Cruz Biotechnology. The ppMEK antibody was purchased from Cell Signaling Technology. Constructs for the expression of recombinant His-MEK1 and His-G1C (constitutively active MEK1) were a gift from N. Ahn (University of Colorado at Boulder, Boulder, CO). Bacterial expression vectors for Raf-239 and Raf-330 were a gift from S.J. Decker (University of Michigan, Ann Arbor, MI). Bacterial expression plasmid for RKIP was a gift from K. Yeung and W. Kolch (Brown University, Providence, RI).

Preparation of mitotic extract, Raf-239, and RKIP

Preparation of cytosol from NRK cells arrested in mitosis and the assay for Golgi complex fragmentation have been described before (Acharya et al., 1998). Raf-239 and RKIP were expressed and purified by the published procedure (Pumiglia et al., 1995; Yeung et al., 1999). The recombinant proteins were dialyzed against KHM buffer containing 5% glycerol and stored at –80°C.

Assay of endogenous RAF activity

The assay was performed as described previously (Bondzi et al., 2000) with the following modifications. 15 μg of Raf-239 RAF1 was added to 350 μg of mitotic cytosol. After a 10-min incubation at 32°C in the presence of ATP, the endogenous RAF1 was immunoprecipitated from the incubation mixture using 2 μg of anti-RAF1 antibody raised against the COOH terminus of the protein. The immunoprecipitated RAF1 (still on beads) was then incubated with 3 μg of recombinant MEK1 and ATP at 32°C. The sample was centrifuged to separate MEK1 (in the supernatant) from RAF1 (on the beads). The supernatant was analyzed by SDS-PAGE followed by Western blotting with anti-ppMEK antibody. The film was scanned and the bands quantitated using the Kodak 1D 3.0 software.

Assay for MEK1 activation

RAF1 and MEKK1 were immunoisolated from 250 μg of mitotic cytosol using 2 μg of anti-RAF1 and anti-MEKK1 antibodies conjugated to Sepharose beads. The beads containing immunoisolated RAF1 and MEKK1 were incubated as described (Bondzi et al., 2000) with MEK1, RKIP, and ATP at 32°C for 10 min. The reaction was then centrifuged to separate beads from the soluble MEK1. The supernatant containing MEK1 was analyzed by SDS-PAGE and Western blotted with anti-ppMEK antibody and analyzed as reported above.

Microinjections

Purified GST and Raf-239 were injected into NRK cells by the same procedures as described previously for GRAPS65-related reagents (Sutterlin et al., 2002).

Effect of Raf-239 and RKIP on Golgi complex fragmentation

350 μg of mitotic extract was pretreated with Raf-239 or RKIP (15 μg/sample) and an ATP regeneration system for 10 min at 32°C. This mixture was added to semi-intact cells and the incubation was performed for 1 h at 32°C. G1C was used at a final concentration of 3 μg/sample. 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 32°C.

Localization of activated MEK1

NRK cells were plated and treated as described before (Sutterlin et al., 2002). Cells were fixed with 4% formaldehyde at 37°C and blocked with PBS containing 0.1% TX-100 and 5% BSA. To reveal the localization of activated MEK1, cells were incubated for 2 h at room temperature in blocking buffer containing 2 μg/ml of ppMEK1 antibody followed by a 1-h incubation with goat anti–rabbit Alexa-546 (1:3,000 dilution). All the other staining procedures were as described (Sutterlin et al., 2002). Epifluorescence microscopy images were acquired with a Nikon microphot-FXA connected to an Olympus CCD camera. Confocal microscope images were captured on a Leika microscope using 488-nm laser excitation for secondary Alexa486, 568 nm for Alexa546, and 351 nm for Hoechst dye. Images for illustrations were captured with a 60 NA objective. Microsoft PowerPoint and Adobe Photoshop 7.0 were used to assemble the figures. To calculate the extent of active MEK1 colocalization with β-COP, five images showing double labeling for active MEK1 and β-COP were selected, the respective Golgi complex areas were selected and cut using Adobe Photoshop. Finally, the NIH Image software (version 1.62) was used to measure the percentage of the β-COP containing pixels and active MEK1. To evaluate the specificity of ppMEK antibody, immunofluorescence blocking buffer containing 0.2 μg/ml of ppMEK antibody were incubated with a 50-M excess of recombinant MEK1 that had been previously phosphorylated as described above. Control sample consisted of ppMEK antibody incubated with nonphosphorylated recombinant MEK1.

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