Phosphorylation of golgin-160 by mixed lineage kinase 3

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Summary

Golgin-160 is a member of the coiled-coil family of golgin proteins, which are proposed to regulate the structure of the Golgi complex. The C-terminal two-thirds of golgin-160 is predicted to form a coiled-coil domain and the N-terminal head domain contains several putative binding domains, regulatory motifs and phosphorylation sites. Recently, it has been demonstrated that caspase-dependent cleavage of the golgin-160 head domain occurs rapidly after induction of apoptosis. The role of golgin-160 phosphorylation and the functional implications for Golgi structure have not been defined. In this study, we investigated the kinase(s) responsible for phosphorylation of golgin-160. Signaling through the small G-protein Rac and mixed-lineage-kinase-3 (MLK3) resulted in increased phosphorylation of golgin-160. The intracellular distribution of MLK3 overlapped with that of golgin-160 and the two proteins could be co-immunoprecipitated. In vitro kinase assays demonstrated that MLK3 directly phosphorylates golgin-160 in the N-terminal head region between residues 96 and 259. Overexpression of MLK3 caused an enhanced caspase-dependent cleavage of golgin-160 at Asp139. Golgin-160 is the first non-kinase substrate of MLK3 identified, and phosphorylation by MLK3 might modulate cleavage of golgin-160 during apoptosis.

Key words: MAP kinase, Phosphorylation, Golgi complex, Golgins, Caspase

Introduction

The Golgi complex plays a central role in post-translational processing and sorting of cargo moving through the secretory pathway in all eukaryotic cells. This ubiquitous organelle has an unusual structure of stacked cisternal membranes, and this unique organization is likely to be important for its function. The regulatory mechanisms that control Golgi-complex structure are still being defined but appear to involve several Golgi-associated proteins called golgins, including giantin, golgin-230, GMAP-210 and GM130 (Infante et al., 1999; Kjer-Nielsen et al., 1999; Linstedt and Hauri, 1993; Nakamura et al., 1997). Some golgins (e.g. giantin) are associated with the Golgi membrane through a C-terminal hydrophobic domain, whereas others (e.g. GM130) are peripheral membrane proteins. Golgins have been implicated in Golgi structure and function, including roles in cisternal stacking and vesicle tethering, and as effectors for the Rab family of small GTPases, which are involved in vesicle transport (Gleeson et al., 1996; Linstedt et al., 2000; Short et al., 2001; Sonnichsen et al., 1998; Valsdottir et al., 2001; Weide et al., 2001).

The Golgi complex undergoes dramatic structural rearrangements during the cell cycle. For example, because the Golgi complex is a single-copy organelle, it must fragment into smaller vesicles during mitosis and then reassemble in each daughter cell upon completion of cell division (Levine et al., 1995). Several studies have revealed potential mechanisms for Golgi disassembly and reassembly during mitosis. GM130 is a Golgi structural protein that interacts with the vesicle tethering protein p115 (Nakamura et al., 1997). During mitosis GM130 becomes phosphorylated, possibly by the cyclin-dependent kinase Cdc2, disrupting its interaction with p115 (Lowe et al., 1998). Upon completion of mitosis, GM130 is dephosphorylated through a mechanism that probably involves protein phosphatase 2A, and this coincides with the reassembly of the Golgi complex (Lowe et al., 2000). Thus, Golgi protein phosphorylation plays an important role in regulating protein-protein interactions and the Golgi structure during mitosis.

Golgin-160 is a coiled-coil protein originally identified as an antigen in certain autoimmune diseases (Fritzler, 1998; Misumi et al., 1997). The protein has several putative functional domains including N-terminal Golgi targeting information, nuclear import and export signals, phosphorylation sites, and an extensive C-terminal coiled-coil domain (Hicks and Machamer, 2002). Recently, it was shown that golgin-160 is cleaved by caspases 2, 3 and 7 at aspartate residues 59, 139 and 311, respectively (Mancini et al., 2000). These cleavage sites are also in the vicinity of several putative phosphorylation sites. Although golgin-160 has been shown to be a phosphoprotein (Misumi et al., 1997), the kinases responsible or the functional consequences of phosphorylation have not been examined.

The mitogen-activated protein (MAP) kinases, which include the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and p38 kinases, are the most widely studied phosphorylation-dependent signaling pathways that mediate the actions of extracellular signals (Lewis, 1998).

The MAP kinases are activated through dual phosphorylation of a threonine / any amino acid / tyrosine (TXY) tripeptide motif by MAP kinase kinases (MKKs). The MKKs are, in turn, activated by dual phosphorylation on serine and threonine residues by MKK kinases (MKKKs), which are often coupled to tyrosine kinase and G-protein-coupled receptors. One family of MKKKs is the mixed-lineage kinase (MLK) family, members of which phosphorylate serine and threonine residues, and activate MKK proteins. Members of the MLK family include the MLK1-MLK3 isoforms, the dual-leucine-zipper-bearing kinase (DLK) and the leucine zipper kinase (LZK) (Gallo and Johnson, 2002; Hirai et al., 1997; Holzman et al., 1994; Ing et al., 1994; Sakuma et al., 1997). MLK proteins are potent activators of the JNK and p38 pathways. For example, MLK isoforms can stimulate the JNK and p38 pathways by direct activation of MKK3, MKK4, MKK6 and MKK7 (Hirai et al., 1997; Merritt et al., 1999; Tibbles et al., 1996). By contrast, DLK appears preferentially to activate the JNK pathway through interactions with MKK7 and not MKK4 (Merritt et al., 1999). Recent evidence also suggests that MLK-3 might regulate cell transformation through MKK1-dependent pathways (Hartkamp et al., 1999). Thus, MLK proteins might regulate multiple MAP kinase pathways by targeting a range of MKK proteins. MLK proteins might also regulate physiological functions by being targeted to specific intracellular structures. For example, DLK has been shown to localize to the Golgi complex but its function there is unknown (Douziech et al., 1999). In addition, overexpressed MLK2 associates with microtubules and might regulate JNK activity and motor protein function (Nagata et al., 1998).

In the studies reported here, we have begun to identify the signaling cascades that regulate golgin-160 phosphorylation and function in Golgi complex structure and response to apoptotic stimuli. Our data demonstrate that MLK3 can directly phosphorylate golgin-160 on the N-terminal head region. In addition, phosphorylation of golgin-160 might regulate caspase dependent cleavage of golgin-160.

Materials and Methods

Reagents

Stock solutions of doxorubicin (60 mM; Sigma, St Louis, MO) in DMSO were diluted to a final concentration of 120 μM. The broad-spectrum cell-permeable caspase inhibitor III (Boc-D-FMK) was purchased from Calbiochem (La Jolla, CA) and kept as a 10 μM stock solution in DMSO. CEP11004 was obtained from Cephalon (West Chester, PA). The green fluorescent protein (GFP) monoclonal antibody was from Roche Diagnostics (Indianapolis, IN), and the polyclonal antibody was purchased from Molecular Probes (Eugene, OR). Hemagglutinin (HA) antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Myc (9E10) antibody was protein-A/Sepharose affinity purified from hybridoma supernatants and eluted with glycine solution pH 3.0. Protein-A/Sepharose was purchased from Amersham/Pharmacia (Piscataway, NJ).

Cell culture

HeLa or CHO cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). A stable HeLa cell line expressing a golgin-160 construct N-terminally tagged with enchanced green fluorescent protein (EGFP) (pEGFP-C1; Clontech) (Mancini et al., 2000) was maintained in DMEM plus 10% FBS and supplemented with 400 μg ml^{-1} geneticin (G418, Sigma).

Transient transfections

Cells were transiently transfected using lipofectamine (Invitrogen Life Technologies) with cDNA (1 μg) encoding a C-terminal Myc (9E10) or EGFP-tagged golgin-160 constructs (Hicks and Machamer, 2002). Proteins were allowed to express for 20-44 hours before harvesting. In some experiments, golgin-160-transfected cells were co-transfected with 0.5 μg cDNA encoding active and inactive versions of Rac-1 (provided by I. Macara, University of Virginia), wild-type and catalytically inactive (K144R) MLK3 (provided by N. Lassam, University of Toronto), V12 Ras, Raf-1-BXB (provided by U. Rapp, University of Würzburg, Germany) or wild-type and catalytically active MKK1 (provided by N. Ahn, University of Colorado). The dual-leucine-zipper-bearing kinase (DLK) expression construct was provided by L. Holzman (University of Michigan).

Immunoblotting

Following treatment, cells were washed twice with cold PBS, lysed with 300 μl tissue lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) and centrifuged at 20,000 ***g*** to clarify lysates. In some cases, proteins associated with post-nuclear proteins were separated from soluble cytosolic proteins by scraping cells into a microfuge tube with extraction buffer (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 minutes. The nuclei were removed after passing cells ten times through a 26-gauge needle and the homogenate was centrifuged at 20,000 ***g*** for 1 minute to pellet the nuclei. The post-nuclear supernatant was further centrifuged for 1.5 hours at 100,000 ***g*** at 6°C to pellet the cytoplasmic membrane proteins and retain the cytoplasmic proteins in the supernatant. Lysates (~20 μg protein) were diluted with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membrane, blocked for 1-2 hours with 5% nonfat dry milk in TBS (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween-20) and incubated with primary antibodies diluted in TBS plus 1% bovine serum albumin (BSA) for 2 hours to overnight. Membranes were washed several times in TBS and incubated with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit antibodies (KPL, Gaithersburg, MD) diluted 1:10,000. Protein immunoreactivity was detected by enhanced chemiluminescence (ECL) (NEN, Boston, MA). To determine the activation level of JNK (see Fig. 2B), the ratio of phosphorylated JNK to total JNK (determined by densitometry) was normalized to the same ratio in the cells transfected with JNK1 alone. To determine the activation level of ERK (see Fig. 2C), the ratio of phosphorylated ERK to α-tubulin (determined by densitometry) was normalized to the same ratio for untreated cells transfected with inactive MLK3.

Immunoprecipitation and phosphatase treatment

Untransfected HeLa cells grown in 35 mm dishes were metabolically labeled with 150 μCi ml^{-1} of ^{35}S-Promix (Amersham/Pharmacia, Piscataway, NJ) in methionine- and cysteine-free medium for 2 hours. After labeling, cells were chased in the absence or presence of doxorubicin (120 μM) for 4 hours. Cells were lysed and immunoprecipitated with anti-golgin-160 C-terminal antibody as described (Hicks and Machamer, 2002). After washing, immunoprecipitations were split in half and mock-treated or treated

with 200 units of λ protein phosphatase (New England Biolabs, Beverly, MA) in the buffer provided by the manufacturer for 30 minutes at 30°C. Samples were washed and resolved by SDS-PAGE on a 7.5% acrylamide gel, followed by fluorography.

Immunofluorescence

HeLa cells were grown on 18-mm diameter cover slips (No. 1, VWR) in 6-cm tissue culture plates. Untransfected or transfected cells were fixed in 4% paraformaldehyde for 5 minutes followed by permeabilization with 0.1% Triton X-100 for 3 minutes. The cells were incubated at room temperature for 1-2 hours in TBS containing 3% BSA followed by incubation for 1 hour with the appropriate primary antibodies. The cells were then washed several times with TBS and incubated with a 1:200 dilution of fluorescein isothiocyanate (FITC) or Texas-Red-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA) and counterstained with DAPI (0.2 μg ml^{-1}; Sigma). Cells were visualized using a Nikon E800 fluorescence microscope, recorded with a Hamamatsu digital CCD camera, and analysed using IP Lab software (Signal Analytics, Vienna, VA).

Protein interaction assays

The interaction between golgin-160 and MLK3 was determined by expressing MLK3 or EGFP-tagged golgin-160 constructs in HeLa or CHO cells. The expressed EGFP-tagged constructs were immunoprecipitated with the GFP antibody and protein-A/Sepharose, and washed extensively with washing buffer (WB; 25 mM Tris pH 7.4, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100). The GFP/protein-A/Sepharose complex was then added to lysates from cells that were transfected with HA-tagged MLK3 wild-type and allowed to incubate for 2 hours. The protein-A/Sepharose and associated proteins were washed in WB and analysed by immunoblotting for GFP and HA following SDS-PAGE.

To assess the interaction between MLK3 and golgin-160 in vitro, a glutathione-*S*-transferase (GST) fusion protein containing the non-coiled-coil domain of golgin-160 (residues 1-393) was expressed in pGEX-2T in *Escherichia coli* BL21-codon plus (Stratagene, La Jolla, CA) and purified using standard methods. MLK3 and Akt-1 (an irrelevant kinase control, kindly provided by S. Gutkind, NIH) were transcribed and translated using the TnT coupled reticulocyte system (Promega, Madison, WI) using ^{35}S-methionine (Amersham), and diluted into binding buffer (100 mM NaCl, 50 mM Hepes, pH 7.2, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT). Glutathione agarose beads were prebound with 5 μg GST or GST/golgin-160(1-393) and then incubated with labeled MLK3 or Akt-1 for 4 hours at 4°C with rotation. Beads were washed three times with binding buffer containing 0.5% Tween-20, and eluted proteins were resolved by SDS-PAGE followed by autoradiography.

Phosphorylation assays

Phosphorylation of golgin-160 by MLK3 in vitro was performed by incubating 1 μg of GST/golgin-160(1-393) or (96-257) with constitutively active MLK3 wild-type immunoprecipitated from transfected HeLa cells. The GST fusion proteins were expressed in *E. coli* BL21-codon plus (Stratagene) using pGEX-2T and purified on glutathione-agarose using standard methods. The kinase buffer (KB) consisted of 25 mM Hepes pH 7.4, 15 mM $MgCl_2$, 1 mM DTT, 20 μM ATP and 10 μCi ^{32}P γ-ATP and the reaction was allowed to proceed for 20-60 minutes at 30°C. The reaction was stopped with 2× SDS sample buffer and the reaction products were separated by SDS-PAGE and either stained with Coomassie Blue or transferred to PVDF membranes for immunoblotting. In other experiments, GFP- or Myc-tagged golgin-160 were immunoprecipitated from cells and incubated with immunoprecipitated MLK3 in KB as described above. Radioactive phosphate incorporation into golgin-160 was analysed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Results

Golgin-160 is phosphorylated by MLK3- and Rac-dependent pathways

A previous study reported that golgin-160 was phosphorylated (Misumi et al., 1997). Although golgin-160 contains putative protein kinase C and casein kinase II sites, specific kinases responsible for golgin-160 phosphorylation have not been identified. Analysis of golgin-160 by SDS-PAGE revealed several forms, with the more slowly migrating forms being sensitive to phosphatase treatment (Misumi et al., 1997). Treatment of cells with the genotoxic agent doxorubicin for 4 hours enhanced the more slowly migrating forms of golgin-160 (Fig. 1A). The more slowly migrating forms of golgin-160 were phosphorylated because they were sensitive to phosphatase treatment (Fig. 1A).

The effects of several signaling proteins on exogenous golgin-160 phosphorylation were examined in co-transfection experiments by assessing golgin-160 gel mobility on immunoblots. The most dramatic change in golgin-160 gel mobility was observed when the MLK3 wild type, which has constitutive kinase activity, was expressed. MLK3 was chosen because it is well characterized and widely expressed in mammalian cells and tissue (Ing et al., 1994). Expression of MLK3 wild type but not catalytically inactive MLK3 enhanced the presence of the slower-migrating form of golgin-160 (Fig. 1B). Other overexpressed signaling proteins that enhanced the gel-retarded form of golgin-160 included an active mutant of Rac1 (v-Rac) (Fig. 1C). By contrast, the expression of several other mutated signaling proteins that are constitutively active, including the dual-leucine-zipper-bearing kinase (DLK), MKK1 (Fig. 1D) and active Ras (data not shown), did not increase golgin-160 phosphorylation as determined by gel retardation. Consistent with previous studies that demonstrate MLK3 is in a pathway regulated by Rac G-protein signaling (Burbelo et al., 1995; Teramoto et al., 1996), these findings are the first to demonstrate that golgin-160 is a downstream target phosphorylated through a Rac1- and MLK3-dependent signaling pathway.

CEP-11004 blocks MLK3-induced golgin-160 phosphorylation

Specific inhibition of MLK activity using indolocarbazole analogs has been shown to protect neuronal cells from apoptosis (Harris et al., 2002; Mota et al., 2001; Murakata et al., 2002). We next tested whether the MLK inhibitor CEP-11004 affected MLK3-induced golgin-160 gel shifts. Cells transfected with MLK3 were incubated in the absence or presence of 0.5 μM CEP-11004 and then harvested for golgin-160 immunoblotting. As shown in Fig. 2A, CEP-11004 inhibited the MLK3-induced gel shift of golgin-160, supporting a role for MLK3 as a golgin-160 kinase. Treatment with CEP-11004 resulted in faster migration of MLK3 itself (Fig. 2A) suggesting that the inhibitor blocks MLK3 autophosphorylation, which occurs in the untreated samples and is an indicator of MLK3 activity (Leung and Lassam, 2001). Treatment with CEP-11004 was specific for MLK,

because neither the MLK-related DLK nor activation of ERK was affected (Fig. 2B,C).

N-terminal region of golgin-160 interacts with MLK3

The relationship between golgin-160 and MLK3 was further examined in protein interaction studies. Cells were transiently transfected with cDNA encoding GFP-tagged golgin-160 proteins and wild-type MLK3. Following immunoprecipitation with anti-GFP antibody, the immune complexes were washed extensively and the GFP-tagged golgin-160 and associated proteins were separated by SDS-PAGE and immunoblotted for MLK3 and GFP. MLK3 showed the most significant interactions with the head region (residues 1-393) and no interactions with the C-terminal coiled-coil region (residues 393-1498) of golgin-160 (data not shown). Therefore, we further identified the region of interaction using additional mutants of GFP-tagged golgin-160 containing regions of the N-terminus that were co-expressed in cells with wild-type MLK3. MLK3 was most efficiently co-immunoprecipitated with golgin-160 residues 96-393, with only background levels precipitated with golgin-160 residues 1-59 or 1-139 (Fig. 3A). By densitometry, the level of MLK3 immunoprecipitated with golgin-160(96-393) was increased 12-fold relative to that immunoprecipitated with golgin-160(1-59) or golgin-160(1-139). Further evidence that MLK3 interacts with golgin-160 was obtained using GST pull-downs. MLK3 (or Akt1, an irrelevant kinase control) were transcribed and translated in vitro in the presence of ^{35}S-methionine. Labeled proteins were incubated with glutathione-agarose pre-bound to either GST alone or GST/golgin-160(1-393). A significant proportion of the MLK3, but not Akt1, was bound to the GST/golgin-160 fusion protein. The results of the GST pull-down experiments suggest that the interaction is likely to be direct, although we cannot rule out the possibility that other proteins in the reticulocyte lysate mediate the interaction.

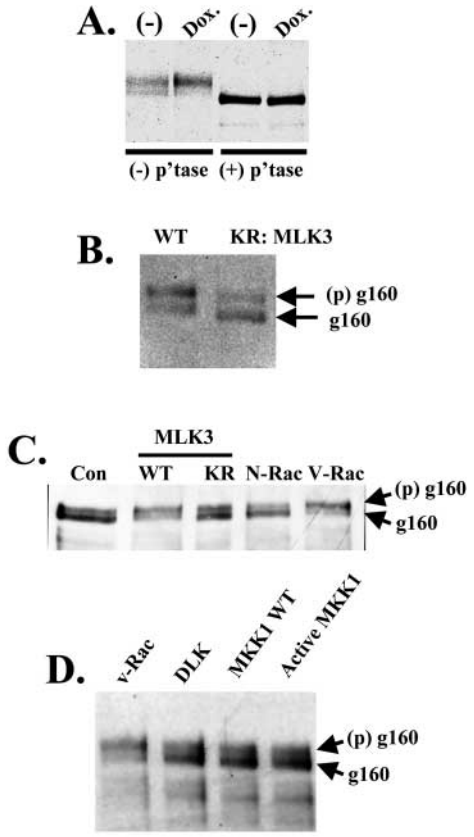

Fig. 1. Phosphorylation of golgin-160 by MLK3- and Rac-dependent pathways. (A) Phosphorylation of endogenous golgin-160 is enhanced in doxorubicin-treated cells. HeLa cells were metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine for 2 hours and chased in normal growth medium in the absence or presence of doxorubicin (120 μM) for 4 hours. Immunoprecipitated golgin-160 was mock-treated or treated with λ protein phosphatase and visualized by fluorigraphy after SDS-PAGE. Elimination of the slower migrating forms in the presence of phosphatase indicates that golgin-160 is a phosphoprotein. Enhanced phosphorylation was observed in doxorubicin-treated cells. (B) Phosphorylation was examined by analysing slower-migrating forms of expressed golgin-160 in CHO cells by immunoblotting, using the anti-Myc (9E10) antibody, for Myc-tagged golgin-160 in cells transfected with wild-type MLK3 (WT) or catalytically inactive MLK3 (KR). The gel was run for 20 hours at 20 mA to enhance the separation of the phosphorylated forms of golgin-160. (C) Myc immunoblot of expressed golgin-160 in cells transfected with cDNA for wild-type (WT) or inactive (KR) MLK3 and inactive (N-) or active (V-) Rac. (D) Myc immunoblot of expressed golgin-160 in cells transfected with cDNA for v-Rac, dual leucine zipper-bearing kinase (DLK), wild-type MKK1 (WT) or a constitutively active MKK1 mutant. The upper and lower arrows in (B-D) indicate the position of the hyperphosphorylated (p) and hypophosphorylated (g160) forms of golgin-160, respectively. Data represent at least four experiments.

MLK3 localizes with golgin-160 in the Golgi region

DLK, a protein kinase related to MLK3, has been localized to the Golgi region (Douziech et al., 1999), and overexpressed MLK2 can interact with microtubules (Nagata et al., 1998). Moreover, MLK3 localization to the centrosomes and perinuclear regions in interphase and mitotic cells (Swenson et al., 2003) suggests that MLK3 and Golgi proteins reside in similar intracellular locations. To examine the intracellular location of MLK3, endogenous or expressed MLK3 was analysed by indirect immunofluorescence microscopy. As shown in Fig. 4A, endogenous MLK3 showed a diffuse cytoplasmic staining pattern as well as juxtanuclear region staining that partially overlapped with the Golgi protein GM-130. Similarly, cells transiently transfected with MLK3 showed intense MLK3 juxtanuclear region staining that also partially overlapped with GM-130 (Fig. 4B). Moreover, the juxtanuclear location of MLK3 overlapped with golgin-160 in stable cell lines expressing GFP-tagged golgin-160 (data not shown). Thus, a proportion of MLK3 is localized to the Golgi region, suggesting that MLK3 and golgin-160 might functionally interact.

MLK3 phosphorylates the N-terminal head region of golgin-160

Golgin-160 contains many potential phosphorylation sites with unknown function. Using EGFP-tagged golgin-160 truncation mutants containing the N- or C-terminal regions, we determined which region of golgin-160 was targeted for

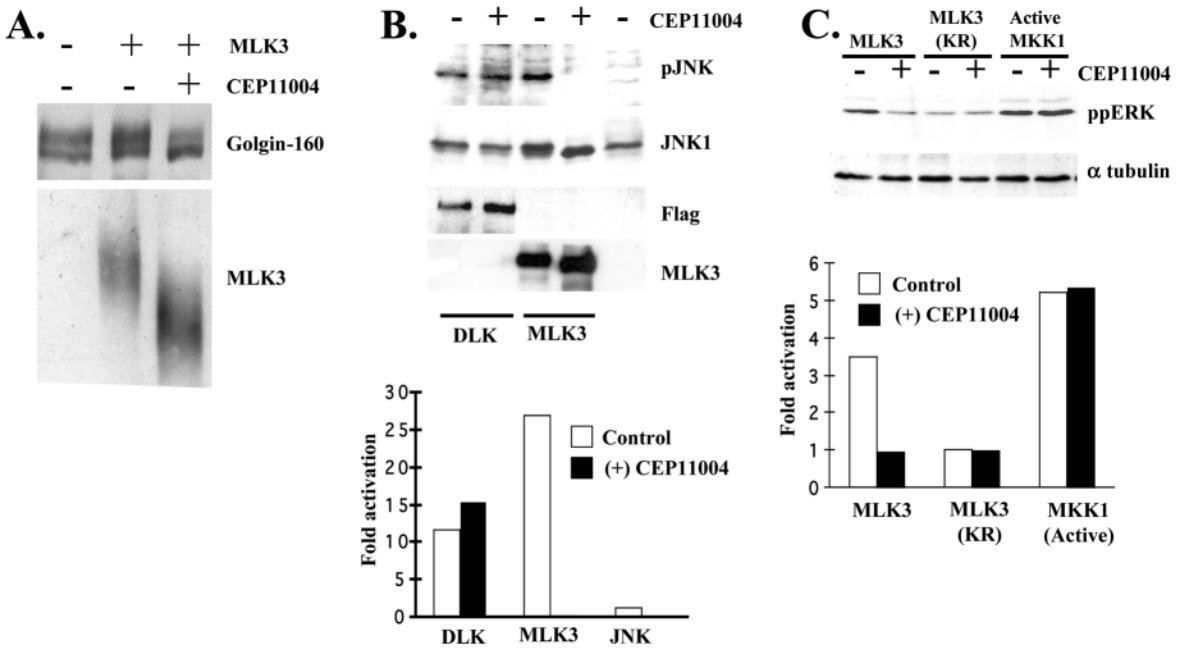

Fig. 2. The MLK inhibitor CEP-11004 blocks MLK3-induced phosphorylation of golgin-160. (A) HeLa cells were transiently transfected with Myc-tagged golgin-160 in the absence or presence of MLK3 and then treated in the absence or presence of CEP-11004 (0.5 μM) during the last 5 hours of the experiment. Protein lysates were separated by SDS-PAGE for 20 hours at 20 mA to achieve separation of phosphorylated forms of golgin-160 (top) and MLK3 (bottom). The gel shift of MLK3 is due to autophosphorylation. (B) CEP-11004 blocks MLK3 but not active DLK. Cells were transfected with HA-tagged JNK1 alone or in the presence of DLK or MLK3 followed by treatment with or without 0.5 μM CEP-11004. Lysates were immunoblotted for phosphorylated JNK1 (pJNK), HA-JNK1, DLK (Flag tag) or MLK3 expression. The activation level of JNK shown in the lower graph was determined by densitometry as described in Materials and Methods. (C) CEP-11004 does not inhibit MKK1. Cells transfected with wild-type or inactive (KR) MLK3 or active MKK1 were treated in the absence or presence of CEP-11004. Lysates were immunoblotted for active ERK (ppERK, top) or α-tubulin (bottom) as a protein loading control. The lower graph shows densitometry analysis of the relative amounts of ppERK in each condition. The activation level of ERK was determined by densitometry, as described in Materials and Methods. Data represent two separate experiments.

phosphorylation by MLK3. Following expression in CHO cells, the EGFP-tagged golgin-160 proteins were immunoprecipitated and incubated for 60 minutes in an in vitro kinase assay with exogenous wild-type MLK3 that was immunoprecipitated from CHO cells. Following incubation, the proteins were resolved by SDS-PAGE and radioactive phosphate incorporated into golgin-160 was examined by phosphorimager analysis. Fig. 5A shows the immunoblots

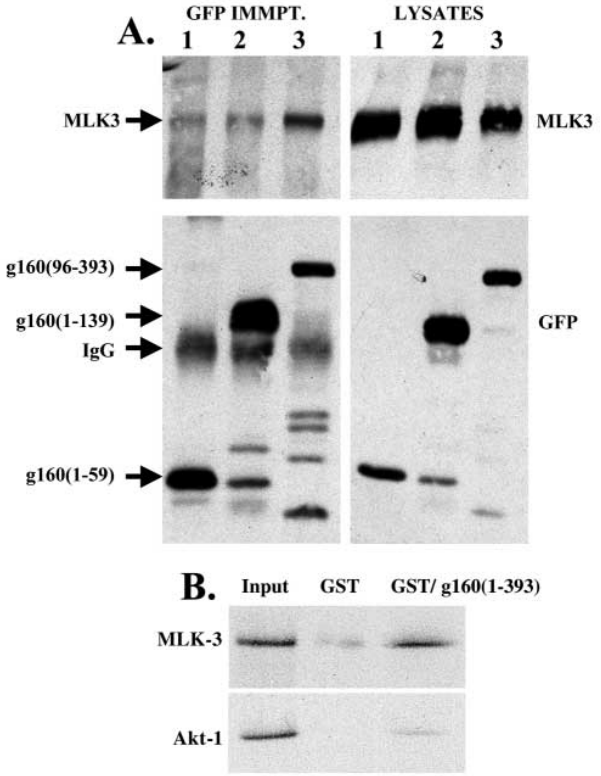

Fig. 3. MLK3 interacts with the N-terminal region of golgin-160. (A) EGFP-tagged golgin-160 deletion mutants containing N-terminal regions spanning amino acids 1-59 (lane 1), 1-139 (lane 2) or 96-393 (lane 3) were transiently expressed with wild-type MLK3 in CHO cells. Following GFP immunoprecipitation, protein complexes were resolved by SDS-PAGE and immunoblotted for MLK3 (top) and GFP (bottom). (Left) The MLK3 and EGFP-tagged golgin-160 proteins in the immunoprecipitations. (Right) The expression of MLK3 and EGFP-tagged golgin-160 proteins in the total cell lysates (10% of the input for the immunoprecipitations). Data represent three separate experiments. (B) MLK3 interacts with GST-tagged golgin-160(1-393). MLK3 or Akt-1 were transcribed and translated in vitro in the presence of ^{35}S-methionine. GST or GST/golgin-160(1-393) pre-bound to glutathione-agarose beads were incubated with the labeled proteins and bound material was resolved by SDS-PAGE and autoradiography. The input is 10% of the starting material added to each pull-down. These data are representative of three independent experiments.

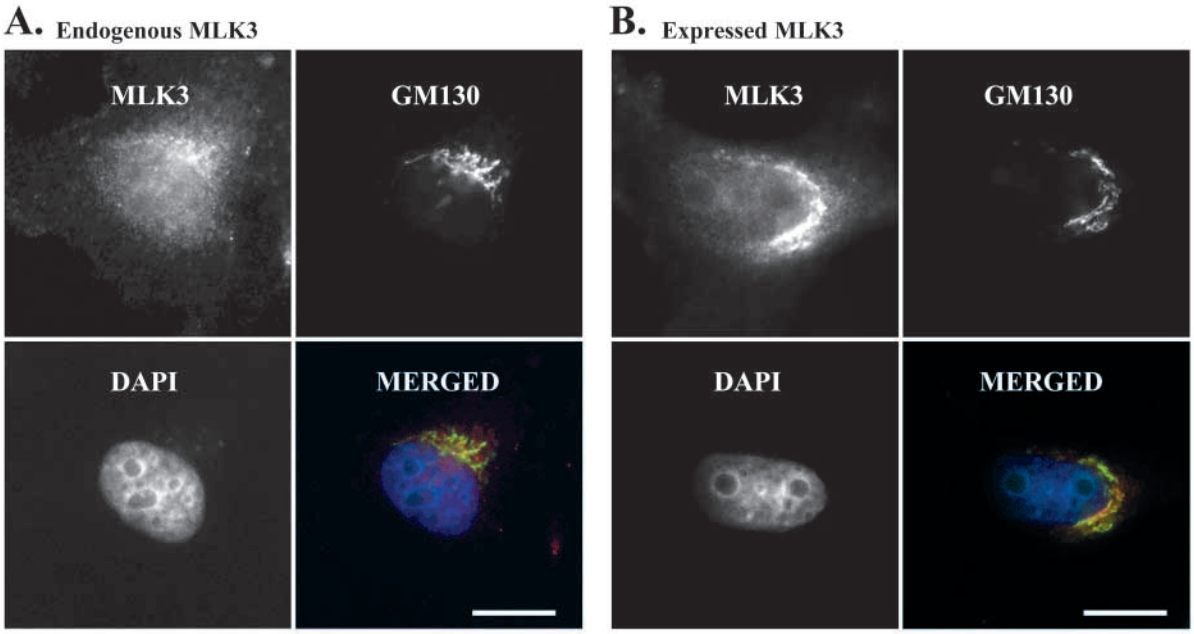

Fig. 4. MLK3 is localized in the juxtanuclear Golgi region. HeLa cells were left untreated or transiently transfected with HA-tagged wild-type MLK3. The localization of endogenous (A) and expressed MLK3 (B) was examined by immunofluorescence in HeLa cells that were co-stained with GM130 as a marker of the Golgi complex. The merged image (lower right) of MLK3 (Texas Red) and GM130 (FITC) demonstrates overlap between the two proteins in the juxtanuclear Golgi region. The nuclear DNA staining with DAPI is shown for reference. Scale bar, 10 μm.

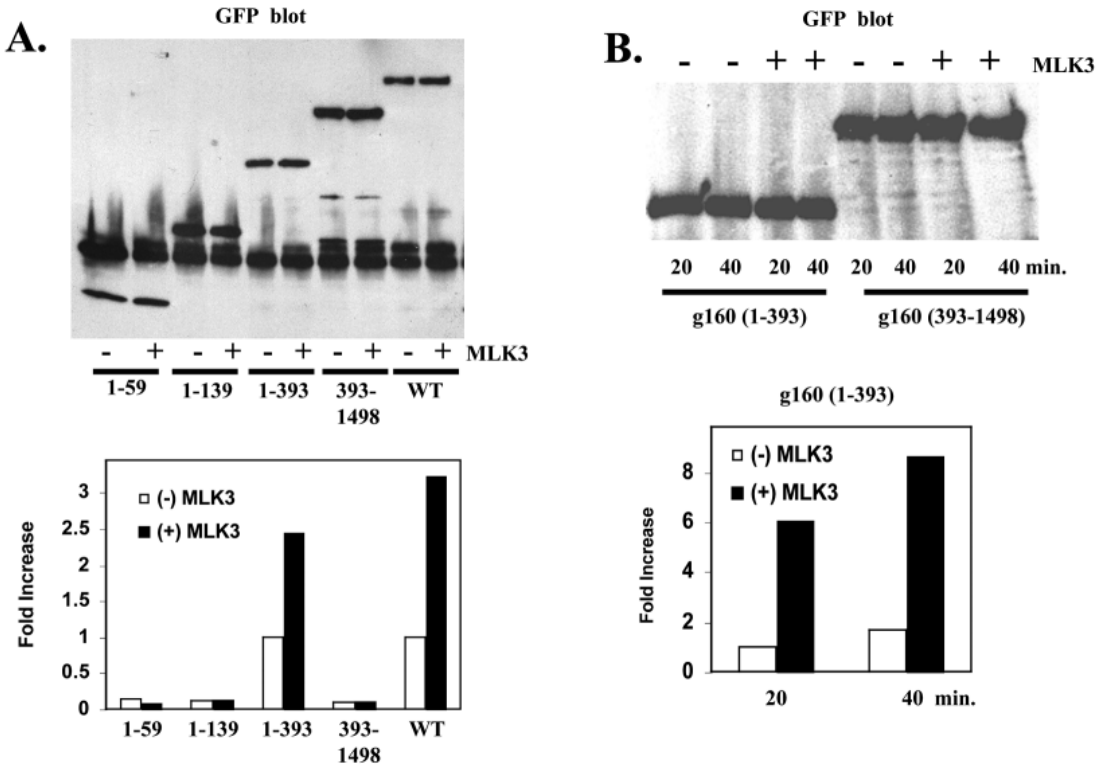

Fig. 5. MLK3 phosphorylates the N-terminal head region of golgin-160. HeLa cells were transfected with GFP golgin-160 mutants encompassing specific amino acid regions of the protein. The golgin-160 proteins were immunoprecipitated and incubated with expressed MLK3 isolated from CHO cells and phosphate incorporation was determined in a kinase assay. (A) (Top) GFP immunoblot of golgin-160 proteins in the kinase reaction mixture after incubation for 60 minutes in the absence or presence of MLK3. (Bottom) Relative phosphate incorporation into golgin-160 wild-type and mutant proteins in the absence (open bars) or presence (closed bars) of MLK3. The phosphorylation of the golgin-160 mutants was normalized to the phosphorylation of golgin-160 wild type in the absence of MLK3, which was set at a value of 1. (B) (Top) Immunoblot of GFP/golgin-160(1-393) and GFP/golgin-160(393-1498) in the kinase reaction mixture after incubation in the absence or presence of MLK3 for 20 minutes or 40 minutes. (Bottom) Phosphate incorporation in the golgin-160(1-393) mutant incubated in the absence (open bars) or presence (closed bars) of MLK3 for 20 or 40 minutes. No phosphate incorporation was observed in the golgin-160(393-1498) fragment, even though the expression levels for both mutant proteins were the same (top). MLK3 increased phosphorylation of golgin-160 wild type and the 1-393 N-terminal mutant by 2.5-6.2 times in three or four separate experiments.

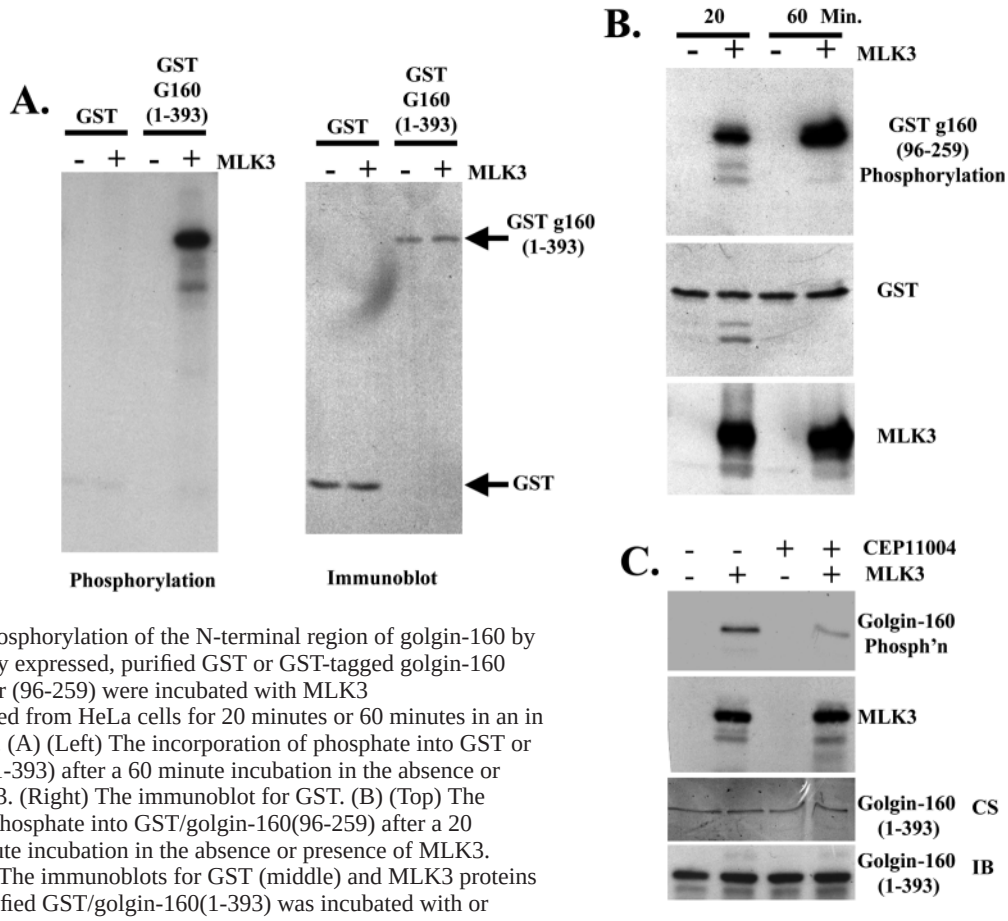

Fig. 6. In vitro phosphorylation of the N-terminal region of golgin-160 by MLK3. Bacterially expressed, purified GST or GST-tagged golgin-160 mutants (1-393) or (96-259) were incubated with MLK3 immunoprecipitated from HeLa cells for 20 minutes or 60 minutes in an in vitro kinase assay. (A) (Left) The incorporation of phosphate into GST or GST/golgin-160(1-393) after a 60 minute incubation in the absence or presence of MLK3. (Right) The immunoblot for GST. (B) (Top) The incorporation of phosphate into GST/golgin-160(96-259) after a 20 minute or 60 minute incubation in the absence or presence of MLK3. (Middle, bottom) The immunoblots for GST (middle) and MLK3 proteins (bottom). (C) Purified GST/golgin-160(1-393) was incubated with or without immunoprecipitated MLK3 in the absence or presence of CEP11004 (0.5 μM). Golgin-160 phosphorylation and MLK3 expression are shown in the top two panels. Total GST/golgin-160(1-393) expression by Coomassie staining (CS) or immunoblotting (IB) are shown in the lower two panels.

of the various EGFP-tagged golgin-160 proteins immunoprecipitated from CHO cells in the absence or presence of immunoprecipitated MLK3. MLK3 induced phosphate incorporation only in the wild-type golgin-160 and the golgin-160(1-393) mutant (Fig. 5A, bottom). The amount of phosphate incorporated into the golgin-160(1-59), (1-139) and (393-1498) fragments was essentially the same as the nonspecific background level (Fig. 5A). These data indicate that MLK3-induced phosphorylation of golgin-160 occurs between residues 139 and 393 of the N-terminus.

The phosphorylation of golgin-160 was also determined by in vitro kinase assay following a shorter incubation period with immunoprecipitated MLK3. The phosphorylation of the golgin-160(1-393) mutant was compared to the golgin-160(393-1498) after incubation for 20 minutes and 40 minutes with MLK3. Similar to Fig. 4A, only the golgin-160(1-393) mutant was phosphorylated in the presence of MLK3, even though the protein expression levels for both golgin-160 protein mutants were similar (Fig. 5B). The slight increase in golgin-160 phosphorylation over time in the absence of MLK3 might suggest the presence of endogenous MLK3 or another kinase that co-immunoprecipitated with GFP/golgin-160 in these assays (Fig. 5B).

To examine further the direct phosphorylation of golgin-160 by MLK3 and to eliminate the presence of golgin-160-interacting kinases, bacterially expressed GST-tagged golgin-160(1-393) or golgin-160(96-259) was incubated with MLK3 immunoprecipitated from HeLa cells. As expected, immunoprecipitated MLK3 phosphorylated GST-tagged golgin-160(1-393) (Fig. 6A). In addition, MLK3 was effective at phosphorylating the smaller golgin-160 mutant protein spanning residues 96-259 (Fig. 6B). MLK3-induced golgin-160 phosphorylation could be inhibited in the presence of CEP-11004, indicating that MLK3 was the primary kinase responsible for phosphorylating golgin-160 (Fig. 6C). These data localize the sites of phosphorylation in the N-terminal head region of golgin-160 by MLK3 to between residues 96 and 259, although it is possible that there are MLK3 phosphorylation sites between 259 and 393. Preliminary mutagenesis experiments suggest that the MLK3 is rather promiscuous in selection of phosphorylation sites in golgin-160 (data not shown).

MLK3 enhances cleavage of golgin-160 at aspartate 139

Three caspase cleavage sites at aspartic acid residues 59, 139 and 311 have been identified on the N-terminal head region of golgin-160 and might be important for golgin-160 regulation

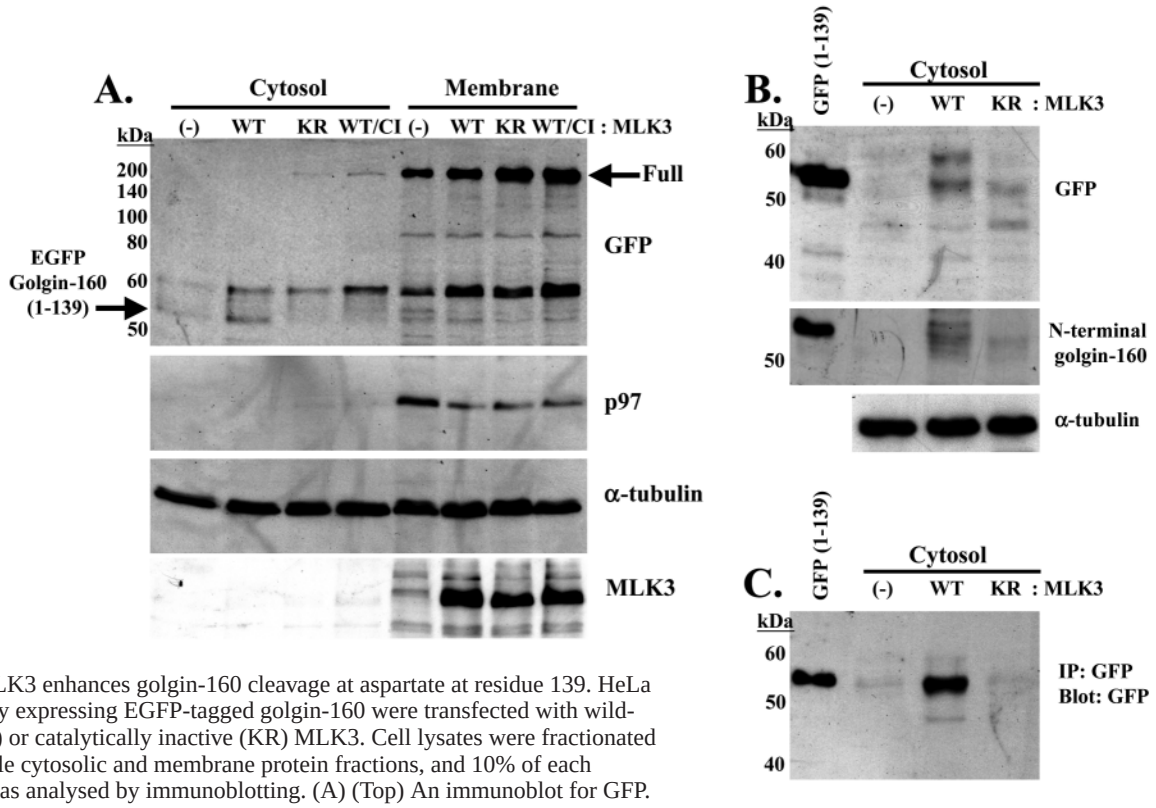

Fig. 7. MLK3 enhances golgin-160 cleavage at aspartate at residue 139. HeLa cells stably expressing EGFP-tagged golgin-160 were transfected with wild-type (WT) or catalytically inactive (KR) MLK3. Cell lysates were fractionated into soluble cytosolic and membrane protein fractions, and 10% of each fraction was analysed by immunoblotting. (A) (Top) An immunoblot for GFP. The arrows indicate the position of full-length EGFP-tagged golgin-160 and EGFP/golgin-160(1-139). The presence of the caspase inhibitor Boc-D-FMK (10 μM) in wild-type MLK3 transfected cells (WT/CI) during the last 24 hours of incubation inhibited production of the golgin-160 cleavage product in the cytosolic protein fraction. Protein loading controls show the expression of p97 golgin and α-tubulin. MLK3 was found primarily in association with the membrane fraction. (B) (Top) A GFP immunoblot of the cytosolic fraction from stable cells transfected with wild-type or inactive MLK3 and compared with an EGFP-tagged golgin-160 mutant spanning residues 1-139. (Middle) Immunoblot of golgin-160 using an antibody specific for the N-terminus. (Bottom) Immunoblot for α-tubulin as a protein loading control. (C) Immunoprecipitation of EGFP-tagged proteins from soluble cytosolic fraction isolated from stable cells transfected with wild-type or inactive MLK3 using a monoclonal GFP antibody. EGFP-tagged proteins were visualized following immunoblotting with a polyclonal GFP antibody. Data represent at least three separate experiments.

of Golgi structure in response to apoptotic stimuli (Mancini et al., 2000). To test whether MLK3 modulates caspase-dependent golgin-160 cleavage, a stable HeLa cell line expressing EGFP-tagged golgin-160 was transiently transfected with wild-type or inactive MLK3. Our transfection conditions alone can cause some degree of caspase activation and apoptosis (data not shown), so the effects of wild-type MLK3 on caspase-mediated golgin-160 cleavage were compared with mock-transfected cells. 44 hours after transfections, the cells were harvested and fractionated into post-nuclear soluble cytosolic and membrane-associated proteins. Wild-type MLK3 expression stimulated the appearance of an EGFP-tagged-golgin-160-reactive species in the soluble cytosolic protein fraction corresponding to the N-terminal portion of golgin-160 cleaved at Asp139 (Fig. 7A). The size of the MLK3-induced fragment migrated at the same molecular weight as expressed EGFP-tagged golgin-160(1-139) (data not shown and Fig. 7B). Production of this fragment was blocked in the presence of a caspase inhibitor (Fig. 7A). In addition, generation of the fragment was not observed in cells expressing a golgin-160 mutant protein that cannot be cleaved by caspases (data not shown). MLK3 was found primarily in association with the membrane protein fractions, supporting its interaction with golgin-160 (Fig. 7A). Immunoblots of cytosolic lysates showed a GFP-reactive species that co-migrated on a SDS-PAGE gel with the EGFP-tagged golgin-160(1-139) mutant primarily in cells transfected with wild-type MLK3 (Fig. 7B). Similarly, an N-terminus-specific anti-golgin-160 antibody also recognized this cleavage product, as well as several lower molecular weight species below the EGFP-golgin-160(1-139) fragment, suggesting further proteolytic cleavage of golgin-160 (Fig. 7B).

To demonstrate further that this GFP-reactive protein was related to golgin-160 cleavage at Asp139, MLK3-transfected cytosolic cell lysates were immunoprecipitated with a monoclonal GFP antibody, immunoblotted with a polyclonal GFP antibody and compared with an EGFP-tagged golgin-160 mutant expressing residues 1-139. This GFP-reactive species in the cytosol, which was induced by wild-type MLK3 expression, was also present in the GFP antibody immunoprecipitations and appeared at the same molecular weight as EGFP-tagged golgin-160(1-139) (Fig. 7C). Our data suggest that signaling through MLK3 could regulate Golgi complex structure by modulating caspase-mediated golgin-160 cleavage during apoptosis.

Discussion

In these studies, we demonstrate that the serine/threonine kinase MLK3 can directly phosphorylate the N-terminal region of golgin-160. MLK3 is an 847 amino acid protein that contains an SH3 domain, two leucine zippers that promote MLK3 dimerization, a Cdc42- and Rac-interactive binding (CRIB) region, a basic region, and a proline rich region (Ing et al., 1994; Leung and Lassam, 1998). Thus, MLK3 contains several protein domains that could be involved in protein-protein interactions. Golgin-160 also contains a proline-rich region and a leucine zipper in the N-terminus (Hicks and Machamer, 2002), which might be important for protein interactions and could explain the observed interactions of golgin-160 with MLK3 (Fig. 3).

The current evidence indicates that MKK3, MKK4, MKK6 and MKK7, the kinase activators of the JNK and p38 families of MAP kinases, are the only substrates that can be directly phosphorylated by MLK3 (Merritt et al., 1999; Tibbles et al., 1996). Activating phosphorylation of MKK3, MKK4, MKK6 and MKK7 occurs on both a serine and a threonine residue within a conserved DSXAKT motif within subdomain 7 (Tournier et al., 1997). However, it has been suggested that MKK1 and MKK2, which are activated by phosphorylation on serine residues within a DSMANS motif and are specific activators of the ERK MAP kinase family, might also be targeted through an MLK3-dependent pathway (Hartkamp et al., 1999). Nonetheless, evidence showing a direct phosphorylation of MKK1 or MKK2 by MLK3 has not been obtained. Because MLK3 is a potent activator of the JNK pathway, it might be expected that MLK3-mediated golgin-160 phosphorylation occurs through JNK activity and not directly by MLK3. However, other potent activators of the JNK pathway, such as overexpression of DLK, did not increase golgin-160 phosphorylation (Fig. 1). Therefore, the current study provides the first evidence that MLK3 can directly phosphorylate and regulate a non-kinase protein. It is also recognized that, in addition to MLK3, other kinases might phosphorylate golgin-160 and regulate protein function. Experiments aimed at identifying the specific sites of golgin-160 phosphorylation are under way.

The function of golgin-160 is unknown, although other golgins are required for normal Golgi structure and function. The N-terminal head region of golgin-160 contains sequences that are likely to be important for protein function. In addition to the caspase cleavage sites at Asp59, 139 and 311 (Mancini et al., 2000), the N-terminal region of golgin-160 contains Golgi targeting information and many putative phosphorylation sites, which are likely to be involved in regulatory processes (Hicks and Machamer, 2002). We have demonstrated that MLK3 phosphorylates golgin-160 between amino acid residues 96-259 (Figs 5, 6). Indeed, we have also demonstrated that exogenous expression of active MLK3 increases caspase cleavage at Asp-139 (Fig. 7). The functional significance of this cleavage is currently being investigated. MLK3-mediated activation of caspase cleavage of golgin-160 might promote disassembly of the Golgi apparatus. Caspase cleavage of golgin-160 could also produce golgin-160 fragments with unique functions (Hicks and Machamer, 2002).

We demonstrate that increased phosphorylation of golgin-160 occurs following short exposures to apoptotic stimuli, such as doxorubicin, suggesting that golgin-160 is an early physiological target of signaling events that promote cell death (Fig. 1). This process might be mediated by doxorubicin-induced activation of several MAP kinase signaling pathways (Yeh et al., 2001). MLK3 and related kinases might be MAP kinase components that are involved in mediating cell death signaling pathways. For example, in neuronal cells, MLK3 activation of caspases and the apoptotic cascade is through a mechanism involving the JNK MAP kinases, c-Jun activation and cytochrome *c* release (Harris et al., 2002; Mota et al., 2001; Xu et al., 2001). Our data suggests that an alternative route for MLK3 in promoting caspase-mediated cell death might be through golgin-160 phosphorylation and regulation of the Golgi complex structure.

Another possible role for MLK3 in regulation of Golgi structure could be during mitosis. During mitosis the Golgi complex undergoes fragmentation in order to ensure stochastic inheritance of this single-copy-number organelle in each daughter cell (Shima et al., 1998). Although the mechanisms that regulate mitotic Golgi fragmentation are largely unknown, this process might be intimately linked to and regulated by microtubules and the formation of the mitotic spindles (Shima et al., 1998). Similarly, MLK3 activation during mitotic transitions might also regulate microtubules, spindle assembly and centrosome function (Swenson et al., 2003). Thus, it is intriguing to postulate that MLK3 might regulate mitotic Golgi disassembly and reformation through a mechanism involving phosphorylation of golgin-160.

In summary, our findings support a unique role for MLK3 in phosphorylation of golgin-160 and potentially enhancing caspase cleavage at Asp139. Golgin-160 is the first non-kinase substrate identified for MLK3. The possibility that phosphorylation by MLK3 modulates the caspase cleavage pattern of golgin-160 is currently being investigated.

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