Identification of a C-terminal Region That Regulates Mitogen-activated Protein Kinase Kinase-1 Cytoplasmic Localization and ERK Activation*

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The C-terminal region of mitogen-activated protein kinase kinase-1 and 2 (MKK1 and MKK2) may function in regulating interactions with upstream kinases or the magnitude and duration of ERK mitogen-activated protein kinase activity. The MKK C-terminal region contains a proline-rich region that reportedly functions in regulating interactions with the Raf-1 kinase and ERK activity. In addition, phosphorylation sites in the C terminus of MKK1 have been suggested to either sustain or attenuate MKK1 activity. To further understand how phosphorylation at the C terminus of MKK1 and protein interactions regulate MKK1 function, we have generated several MKK1 C-terminal deletion mutants and examined their function in regulating MKK1 localization, ERK protein activation, and cell growth. A deletion of C-terminal amino acids encompassing two putative α-helices between residues 330 and 379 caused a redistribution of mutant MKK1 proteins to membrane compartments. Immunofluorescence analysis of MKK1 mutants revealed a loss of homogenous cytosolic distribution that is typically observed with MKK1 wild type, suggesting this region regulates MKK1 cellular localization. In contrast, MKK1 C-terminal deletion mutants localized to various sized punctate regions that overlapped with lysosome compartments. ERK activation in response to constitutively active Raf-1 or growth factor stimulus was attenuated in cells expressing MKK1 C-terminal deletion mutants. This could be partly explained by the inability of Raf-1 to phosphorylate MKK1 C-terminal deletion mutants even though the phosphorylation sites were intact in these mutants. Finally, we show that cells expressing MKK1 C-terminal deletion mutants displayed characteristic patterns of apoptotic cell death and reduced cell proliferation. These findings identify a novel C-terminal region between amino acid residues 330 and 379 on MKK1 that is necessary for regulating the cytoplasmic distribution and subsequent ERK protein activation necessary for cell survival and viability.

The mitogen-activated protein (MAP)1 kinase kinase-1 and 2 proteins (MKK1 and MKK2) are currently the only known direct activators of the extracellular signal-regulated kinases-1 and 2 (ERK1 and ERK2) (1). The regulation of MKK1 has been extensively studied and requires phosphorylation at serines on position 218 and 222 by Raf or Mos protein kinases for full activation (2). Furthermore, regions within the N terminus region of MKK1 are important for determining catalytic activity, nuclear export, and interactions with ERK proteins (3–5). MKK proteins are maintained primarily in the cytosol through a N-terminal nuclear export sequence (6). However, MKK1 has been shown to translocate to the nucleus following mitogenic stimulation or during G2/M cell cycle transitions (4, 5).

An additional level for MKK1 regulation may occur through C-terminal phosphorylation sites at residues Thr-286, Thr-292, and Thr-386. Several known kinases, including ERKs and Cdc2, and possibly unknown kinases may be responsible for phosphorylation of MKK1 at these sites. The consequence of Thr-286, Thr-292, or Thr-386 phosphorylation is the subject of controversy. In some studies, Thr-292 phosphorylation has been shown to allow prolonged MKK1 activation in response to serum stimulation (7). In contrast, phosphorylation at Thr-286, Thr-292, or Thr-386 by ERK or p34 Cdc2 kinase may also function in the negative regulation of MKK1 activity (8, 9). Furthermore, phosphorylation of Thr-386 has been reported to regulate MKK1 interactions with the Grb2-like adaptor protein, Grb10, following insulin stimulation and may play a role in modulating ERK pathway signaling (10). Thus, it is still not clear how phosphorylation of these residues modulates ERK pathway signaling.

Both MKK1 and MKK2 contain a proline-rich region located in the C terminus between amino acids 262 and 307, which are reported to function in promoting interactions with Raf-1 (7). However, other studies indicate that, although the proline-rich region is important for obtaining full ERK activation following stimulation, removal of this region does not affect MKK and Raf-1 binding (11). Both MKK1 and MKK2 isoforms can phosphorylate ERK proteins to a similar degree (1). However, some differences may exist between MKK1 and MKK2, in regard to both ERK activation and physiological function. For example, MP-1 has been identified as a MKK1-specific binding partner that facilitates sustained activation of MKK1 and ERK1 (12). In addition, MKK1 and MKK2 may display some differences depending on the cellular response. In one example, MKK2, and not MKK1, has been reported to be necessary for cell cycle transitions through G2 and mitosis in cells exposed to ionizing radiation (13). In these studies, cells expressing a dominant negative MKK2 mutant failed to arrest in response to DNA damage.

The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; MKK, mitogen-activated protein kinase kinase; EGF, epidermal growth factor; HA, hemagglutinin; DAPI, 4',6-diamidino-2-phenylindole; TBS, Tris-buffered saline; PMA, phorbol 12-myristate 13-acetate.
damage, suggesting MKK2 activation is involved in maintaining a genotoxically induced G2 checkpoint. In addition, expression of a dominant negative MKK1 in 3T3 cells was reportedly sufficient to cause a delay in cell cycle progression through G2 phase and into mitosis (14). In both of these examples, a corresponding role for ERK protein activation in these responses could not be demonstrated, suggesting that MKK can function independent of ERK in some situations. Thus, MKK1 and MKK2 isoforms may function independently depending on the conditions and interact with novel unknown substrates.

In the absence of structural data derived from x-ray crystallography and nuclear magnetic resonance studies, the relationship between MKK protein structure and function has yet to be determined. However, studies using deuterium exchange methodology to estimate the flexibility of specific regions on the MKK1 protein have given some insight to its structure-function relationship (15). Based on this study and the amino acid sequence, MKK1 is predicted to contain many of the structural features common to known kinases. For example, the N- and C-terminal regions represent opposing lobes that may show flexibility to allow exposure of substrate binding sites and phosphorylation of the activation lip. Furthermore, changes in flexibility in the C-terminal region of MKK1 opposite N-terminal substrate binding regions could potentially influence substrate binding and downstream activation (15).

In these studies, we examined several MKK1 C-terminal deletion mutants expressed in cells to further define how this region regulates MKK1 function. We present evidence that a C-terminal region outside of the proline-rich domain and not containing any of the known C-terminal phosphorylation sites is necessary for maintaining MKK1 protein in a soluble cytosolic localization. In addition, we show that this C-terminal region is important in allowing MKK1 to be activated by Raf-1 and cell proliferation. Thus, we have identified a novel C-terminal region on MKK1 that is necessary for ERK pathway activation and cell viability.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Hela cells were maintained in complete medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum), supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were transfected using 1 μg of cDNA and 5 μl of LipofectAMINE reagent (Life Technologies, Inc.) in OptiMEM (Life Technologies, Inc.) for 4 h. Transfection efficiencies were ~30% as estimated by green fluorescence protein expression. Cells were typically harvested 18–24 h after transfections. Cell proliferation assays were done by seeding the same number of cells within an experiment on 35-mm culture dishes, transfecting with MKK1 constructs, and counting cells after 20–22 h of expression using trypan blue staining or MTS tetrazolium reduction to Formazan (CellTiter 96® AQmes® nontoxic, nonradioactive cell proliferation assay, Promega).

**Reagents**—Monoclonal antibodies recognizing α- or γ-tubulin and phosphorylated ERK1/2 (pT183, pY185) were purchased from Sigma. Polyclonal antibody recognizing phosphorylated MKK1/2 (pS217, pS221) was purchased from New England Biolabs. Polyclonal antibodies recognizing MKK1 (C-18) and ERK2 (C-14) and the monoclonal antibody recognizing hemagglutinin tag (HA) were purchased from Santa Cruz Biotechnology. Lysosome staining was performed on cells during the last 1 h in culture using 50 nM LysoTracker red reagent (Molecular Probes, L-7528) that was kindly provided by Dr. Carolyn Machamer (The Johns Hopkins University, Baltimore, MD). Following incubation with LysoTracker reagent, cells were processed for immunofluorescence and examined following fixation as described below. Raf-1 87× EB DNA construct was kindly provided by Dr. Ulf Rapp (University of Würzburg, Würzburg, Germany). MKK1 C-terminal Deletion Mutants—Full-length MKK1, cloned into the BamHI and HindIII sites C-terminal to the HA tag on the pMCL vector, was used as the template and kindly provided by Dr. Natalie Ahn (University of Colorado, Boulder, CO) (16). The N-terminal PCR primer containing the BamHI site 5′-GGATACATCAATGACACCTCCTAC (MKK1 d330), and 5′-AGACTTGTACCGATTGGTGGGAGCA (MKK1 d379). The MKK1 PCR products were gel extracted and subcloned into the BamHI and HindIII sites of pMCL. The MKK1 mutant with amino acids deleted between the full-length MKK1 template using the primers 5′-ACTGAAACCTCCACTGTTGCGATTTTGGAG and 5′-pCTTAACGCGCCCGACACCACTACATGGC and the MKK1 product was religated.

**Immunoblotting**—Untransfected and transfected cells were washed twice with cold phosphate-buffered saline, lysed with 300 μl of tissue lysis buffer (20 μg/ml Tris, pH 7.4, 157 mM NaCl, 2 μl EDTA, 1% Triton X-100, 0.1% β-mercaptoethanol, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine), and centrifuged at 15,000 rpm to clarify lysates. In some experiments, a final concentration of 0.1% SDS was added to the lysis buffer to promote extraction of membrane proteins. Lysates (~20 μg of protein) were diluted with an equal volume of 2× SDS-sample buffer and resolved by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane, blocked for 1–2 h with 5% nonfat dry milk in Tris-buffered saline (TBS: 50 mM Tris, pH 7.5, 0.15 mM NaCl, and 0.1% Tween 20), and incubated with primary antibodies diluted in TBS + 1% bovine serum albumin for 2 h to overnight. Membranes were washed several times in TBS and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson Immunoresearch, diluted 1:10,000). Protein immunoreactivity was detected by enhanced chemiluminescence (PerkinElmer Life Sciences).

**Cell Fractionations**—Cytosolic and nuclear proteins were separated as described previously (17, 18). Briefly, cells were harvested by scraping into a microcentrifuge tube with extraction buffer containing 10 mM Hepes, pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride followed by incubation on ice for 15 min. Passing cells through a 26-gauge needle 10 times was performed to isolate nuclei. The homogenate was centrifuged at 14,000 rpm for 1 min to pellet the nuclei and generate a postnuclear supernatant. Nuclear proteins in the nuclei pellet were extracted by frequent vortexing in 20 mM Hepes, pH 7.4, 1.5 mM MgCl2, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. The postnuclear supernatant fraction was further centrifuged for 1.5 h at 100,000 × g at 6 °C. The supernatant containing soluble cytosolic proteins and the pellet containing cytoplasmic membrane proteins were re-extracted with SDS-PAGE sample buffer. The expression of MKK1 proteins in the cytosolic and nuclear fractions was analyzed by immunoblotting following SDS-PAGE as described above.

**Immunofluorescence**—Cells were grown on round glass coverslips in 6-cm plates and transfected as described above. Coverslips were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 5 min and permeabilized with 0.1% Triton X-100 for 2 min. Localization of MKK1 proteins was identified by immunostaining for the HA tag and counterstained for cellular DNA with 4′,6-diamidino-2-phenylindole (DAPI, 0.2 μg/ml in phosphate-buffered saline). Cells were identified using a Nikon E800 fluorescence microscope and Hamamatsu CCD camera and processed with IPLab software. The HA-MKK1 pattern of staining in cells was examined in more than 100 cells for MKK1 wild type, MKK d330–379, and MKK d296. Normal cells displayed a diffuse homogeneous HA pattern throughout the cytoplasm and sometimes in the nucleus. The punctate pattern of staining was characterized as many distinct spots located throughout the cytoplasm. The third pattern of staining was observed in nuclei. The punctate pattern of staining was characterized as many distinct spots located throughout the cytoplasm.
MKK1 wild type or MKK d379 showed a typical diffuse staining pattern throughout the cytoplasm and nucleus (Fig. 3, A and B). The nuclear localization of MKK1 could be because of high protein expression levels as observed in other systems (22). In contrast, MKK d330–379, MKK d330, and MKK d296 deletion mutants all showed a dramatic localization to varying sized punctate structures often located to the perinuclear region but also scattered throughout the cytoplasm (Fig. 3, C–E). The percentage of transfected cells displaying a normal (Fig. 3, A and B), punctate (Fig. 3F), or aggregate (Fig. 3, C and D) HA staining patterns was determined in cells expressing MKK1 wild type and the d296 mutant. Approximately 75–80% of the transfected cells expressing the MKK1 d296 mutant showed a punctate or aggregate HA staining pattern compared with none in cells transfected with MKK1 wild type (Fig. 3F).

Similar results were obtained with cells expressing d330 and d330–379 mutants (data not shown). To further demonstrate that deletion of a MKK1 C-terminal region between residues 330 and 379 targeted MKK1 to cellular membranes, cell lysates were partially fractionated into cytosolic, membrane, and nuclear proteins as described previously (18). As shown in Fig. 3G, the MKK d330–379 mutant was found exclusively in the membrane protein fraction compared with MKK1 wild type, which could be found in both cytosolic and membrane protein fractions. We further confirmed the validity of this method for generating cytosolic and membrane proteins by immunoblotting for endogenous MKK1 and ERK2, the Golgi complex protein GM130 as an intracellular membrane marker, and the nuclear protein topoisomerase IIa. As shown in Fig. 3H, endogenous MKK1 is found primarily found in the cytosolic fractions, whereas endogenous ERK2 is found primarily in the cytosolic fraction but also in the membrane and nuclear fractions. In contrast, the intracellular membrane protein, GM130, and the nuclear protein, topoisomerase IIa, are found only in the membrane and nuclear fractions, respectively (Fig. 3H).

The irregular pattern of staining with the HA-MKK1 deletion mutants was similar to nonhomogenous size or distribution that is characteristic of lysosome organelles (29). To confirm localization of MKK1 mutants to lysosomes, transfected cells were incubated with a commercially available probe that is specific for acidic organelles, such as lysosomal compartments (LysoTracker dye, see “Experimental Procedures”). MKK1 wild type showed a typically diffuse HA staining pattern and no apparent overlap with the lysosome marker (Fig. 4A). In comparison, the aggregate and punctate staining pattern shown in two views of MKK d296 mutants displayed some degree of overlap with lysosome compartments (Fig. 4, B and
The aggregate HA staining pattern showed higher levels of lysosome co-localization compared with the punctate HA staining pattern and could indicate different stages of MKK1 processing and targeting to lysosomal compartments (Fig. 4, B and C). These data indicate that the C-terminal region of MKK1 functions in maintaining the protein in a soluble cytoplasmic location and prevents targeting to lysosomes.

MKK1 C-terminal Deletion Mutants Enhance Markers of Apoptosis—In some cases, visual inspection of cells expressing MKK d330 or MKK d296 showed a larger number of detached floating cells compared with MKK1 wild type expressing cells and suggested these cells were susceptible to cell death (data not shown). To examine whether the C-terminal region is involved in cell viability, MKK1 expression levels
were examined in adherent and floating cells lysed in the presence of 0.1% SDS. Immunoblot analysis for HA-MKK1 shows the expression levels for wild type and C-terminal mutants in the adherent cell lysates (Fig. 5, A and B, top panel). In Fig. 5A, the expression of MKK1 wild type (lane 2) was found exclusively in the adherent cells, whereas high levels of expression for MKK d330 and MKK d296 were also apparent in the floating cell lysates (Fig. 5A, top panel, lanes 4 and 5). MKK d379 also showed some expression in the floating cells, although at lower levels compared with the other C-terminal deletion mutants (Fig. 5A, lane 3). Similarly, high levels of MKK d330–379 expression were apparent in floating cells compared with MKK1 wild type (Fig. 5B). The total number of floating cells was not apparently much different between any of the conditions, as evidenced by similar levels of α-tubulin, and suggested that the transfection conditions alone, not surprisingly, cause some cell toxicity (Fig. 5, A and B, lower panel). However, the high levels of MKK1 expression in the floating cells of the cells expressing C-terminal deletion mutants indicated these cells were more susceptible to cell death as compared with cells expressing MKK1 wild type.

The role of the C-terminal region of MKK1 is supporting cell viability was further examined by evaluating changes in nuclear structure as an indicator of early apoptotic events (19). Nuclei from untransfected and transfected cells were examined for normal round appearance and abnormal shapes characterized by the bilobed appearance shown in the DAPI staining in Fig. 3D. Cells transfected with MKK1 wild type showed less than 10% of the cells contained abnormally shaped nuclei (Fig. 5C). Similar results were obtained with cells expressing MKK d379 (data not shown). In contrast, expression of MKK d330–379, d330, and d296 mutants resulted in a significantly higher percentage of transfected cells that contained abnormal nuclei structure (Fig. 5C). Adjacent untransfected cells under these conditions also showed increased number of cells containing abnormally shaped nuclei as compared with untransfected cells adjacent to cells transfected with MKK1 wild type (Fig. 5C).

The previous data indicated that expression of MKK1 lacking C-terminal amino acid residues from 330 to 379 was toxic to cells. To further establish the effects of MKK1 mutants on cells, DNA fragmentation as a marker of apoptosis was examined. Fragmented DNA was fluorescently labeled (see “Experimental Procedures”) in cells expressing MKK1 wild type, d330–379, d330, or d296, and the percentage of positively labeled cells were calculated. Fig. 5D shows an image of apoptotic cells found in cells transfected with MKK d296 (bottom right panel) or MKK1 wild type (upper right panel). Cells expressing MKK d330–379, d330, or d296 showed 2–2.5 times more apoptotic cells as compared with cells expressing MKK1 wild type (Fig. 5E). These findings indicate that the C-terminal region of MKK1 spanning amino acids 330–379 functions in promoting cell viability and protects against cell death.

Expression of MKK1 C-terminal Deletion Mutant Causes Inhibition of Cell Growth.—The effects of the MKK1 C-terminal region on cell proliferation was examined in cells transiently transfected with MKK1 wild type and MKK1 C-terminal deletion mutants. In agreement with the data presented above, the proliferation of cells expressing the MKK1 C-terminal deletions d330–379 or d330 for 20 h was inhibited as measured by direct cell counting (Fig. 6A) or by the conversion of MTS tetrazolium to formazan as an indicator of cell viability (Fig. 6B). Thus,
these data point to an integral role for the C-terminal region of MKK1 in promoting cell viability.

**MKK1 Activation Is Inhibited in Proteins Lacking the C-Terminal Region**—To examine a possible mechanism to explain the loss of cell viability with the MKK1 C-terminal deletion mutants, MKK1 and ERK activation was tested in transfected cells following growth factor treatment or co-expression of constitutively active Raf-1. First, cells expressing HA-MKK1 and HA-ERK2 were stimulated with phorbol ester (PMA) or with epidermal growth factor (EGF) to activate the MKK/ERK pathway. Both PMA and EGF activated ERK in cells transfected with MKK1 wild type or d379 (Fig. 7A). In contrast, cells expressing MKK d330 or d296 showed marked attenuation of PMA or EGF-induced ERK activation (Fig. 7A). The higher basal activity of MKK d379 compared with MKK1 wild type is consistent with the previously reported phosphorylation of threonine 386 acting as a negative inhibitor of MKK1 activity (8). Similarly, cells expressing the MKK d330–379 mutant were also defective in ERK activation in response to EGF stimulation (Fig. 7C).

To test whether the diminished ERK activation was the result of defects in MKK interactions with the upstream Raf-1 kinase, cells were transfected with MKK1 wild type or MKK1 deletion mutants in the absence or presence of constitutively active Raf-1. First, cells expressing HA-MKK1 and HA-ERK2 were stimulated with phorbol ester (PMA) or with epidermal growth factor (EGF) to activate the MKK/ERK pathway. Both PMA and EGF activated ERK in cells transfected with MKK1 wild type or d379 (Fig. 7A). In contrast, cells expressing MKK d330 or d296 showed marked attenuation of PMA or EGF-induced ERK activation (Fig. 7A). The higher basal activity of MKK d379 compared with MKK1 wild type is consistent with the previously reported phosphorylation of threonine 386 acting as a negative inhibitor of MKK1 activity (8). Similarly, cells expressing the MKK d330–379 mutant were also defective in ERK activation in response to EGF stimulation (Fig. 7C).

**DISCUSSION**

In these studies, we demonstrate that the amino acids sequence between residues 330 and 379 in C terminus of MKK1 is important for maintaining MKK1 as a soluble cytoplasmic protein. Importantly, this region appears to protect the viability of cells by protecting the integrity of the Raf/MKK1/ERK signaling pathway. Other studies have reported that the proline-rich region located between amino acids 362 and 307 of MKK1 and MKK2 may function in regulating interactions with c-Raf-1 (21). Although our studies did not attempt to reproduce these findings, we provide evidence that C-terminal amino acid residues from 330 to 379, which are outside of the proline-rich domain of MKK1, are also important for Raf-1 coupling. In addition, this region between residues 330 and 379 does not contain any of the putative C-terminal regulatory phosphorylation sites. Thus, the C terminus of MKK1 may use multiple mechanisms for regulating protein interactions and ERK signaling necessary for cell growth and survival.

Based on the requirement for SDS to extract MKK1 mutant proteins and immunofluorescence data (Figs. 2 and 3), our findings indicate that MKK1 proteins lacking amino acids 330–379 cannot be activated by Raf-1 because of the inability for the two proteins to physically interact. The punctate MKK1 staining pattern shown in Fig. 3 with the C-terminal deletion mutants is likely a form of MKK1 that is inaccessible to interactions with Raf-1. Further evidence to support these findings is presented by the attenuated phosphorylation of MKK d330–379, d330, and d296 deletion mutants in response to constitutively active Raf-1 as compared with MKK1 wild type or d379 mutant (Fig. 7B). The amino acid residues between 330 and 379 on MKK1 may also function in facilitating a proper structural conformational on MKK1 that allows the proper protein-protein interactions between Raf-1 and subsequent phosphorylation. Thus, MKK1 activity may be regulated through mechanisms that involve both changes in intracellular localization and structural constraints.

**DISCUSSION**

In the absence of a crystallographic structure for MKK1, it remains to be determined how the C-terminal region could function in regulating interactions with upstream activators or substrates. Nonetheless, preliminary predictions of the structure of MKK1 indicate that the C-terminal region between residues 330 and 379 contains two putative a-helices that may be in contact with N-terminal regions that are important for activity (15). Furthermore, changes in the flexibility of the C-terminal region of MKK1 during activation, as determined by deuterium exchange experiments, may affect N-terminal interactions with ERK substrate or ATP binding (15). Future studies aimed at characterizing the structural components of MKK proteins will be necessary for determining the functional significance of the C-terminal region in regulating MKK1 activity.

The targeting of MKK1 C-terminal deletion mutants to lysosomal compartments and the enhanced stringency required for protein extraction suggest a unique function for this region in maintaining MKK1 proteins as soluble cytoplasmic proteins. One potential role for the C-terminal region of MKK1 is to mask a lysosomal targeting sequence that functions in regulating MKK1 stability and turnover. Two sequences that are recognized to target proteins to lysosome, endosome, or trans-Golgi compartments include the YXXO motif (where Y is tyrosine, X is any amino acid, and O is a bulky hydrophobic residue) or dileucine (LL) signals (24). Interestingly, MKK1 contains such a sequence Y130GAF133 in a putative N-terminal a-helix within the ATP binding region. Whether this sequence functions in directing the intracellular MKK1 localization and is regulated by interactions with C-terminal sequences is currently under investigation.

Other examples exist that demonstrate a role for ERK pathway proteins in regulating substrate protein localization. The ERK substrate p90<sup>66k</sup> has been shown to regulate NF-kB nuclear targeting by phosphorylating IxB-α, stimulating IxB-α degradation, and allowing the exposure of a NF-kB nuclear localization signal (25). Similarly, the C-terminal region of MKK1 could function in coordinating protein interactions that direct MKK1 to the lysosome for degradation. Protein aggregation has been postulated as a mechanism for targeting proteins to the lysosome (26). The C-terminal region of MKK1 may also...
function in preventing protein aggregation and lysosomal degradation. This is supported by our immunofluorescence data, which show a high level of co-localization between aggregated MKK1 C-terminal deletion mutant proteins and lysosomal compartments (Fig. 4).

The mechanisms responsible for targeting MKK1 proteins lacking amino acids 330–379 to intracellular membranes and determining whether MKK1 mutants are packaged within lysosome/endosome vesicles or are in tight association with the cytoplasmic face of the lysosomal/endosomal membrane are not known. Recently, it has been suggested that ERK and MKK proteins may be targeted to the cytoplasmic face of endosomal/lysosomal compartments through the MKK1-binding protein, MP1, and a novel p14 scaffolding protein (27). This targeting of MKK/ERK components to lysosome or other intracellular membranes is suggested to be another mechanism for regulating compartmentalized ERK pathway activity. Another example of compartmentalized activation of the MKK/ERK pathway is suggested by MKK1’s involvement in regulating mitotic Golgi fragmentation through a process involving proteolytic processing (28). These studies reported that MKK1 activity is required for mitotic Golgi fragmentation and suggested that the phosphorylated active form of MKK1 undergoes a conformational change during mitosis that makes MKK1 susceptible to limited proteolysis. Although a corresponding ERK activity was not shown to be required in these studies, work from our laboratory has recently reported that ERK proteins phosphorylated only on the tyrosine within the threonine-glutamate-tyrosine tripeptide activation motif associate with the Golgi complex during G2/M transitions and regulate Golgi structure through a kinase-independent mechanism (18). Whether limited proteolysis of MKK proteins directs active MKK1 to membranes to generate partially phosphorylated ERK proteins on the Golgi complex during mitotic transition remains to be determined.

Finally, MKK proteolysis may also function in response to apoptotic signals. A recent report describes the generation of a caspase-dependent 33-kDa fragment from MKK1 or MKK2, which may function in down-regulating ERK activity and cell survival, in cells treated with vitamin D3 to induce apoptosis (29). The protease that targets MKK proteins and cleavage site under these conditions has yet to determined. Thus, mitotic and apoptotic pathways may use similar mechanisms of regulated MKK proteolysis as a means of modulating ERK activity and signaling pathways required for cell viability.

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