Control of the Cell Morphology and the S Phase Entry by Mitogen-activated Protein Kinase Kinase

A REGULATORY ROLE OF ITS N-TERMINAL REGION

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The mitogen-activated protein kinase kinase (MAPKK)/MAP kinase (MAPK) cascade plays an important role in the growth control of mammalian cells. We have found that expression of constitutively active MAPKK induces rapid morphological changes of fibroblastic cells, which are accompanied by disruption of stress fibers and disappearance of focal adhesions. These changes took place under the conditions that inhibited cellular Ras function, suggesting a linkage between the MAPK cascade and the control of cell morphology. We further show that constitutively active MAPKK can induce expression of endogenous Fos protein, an immediately early gene product, and cause the S phase entry of G0-arrested cells. Finally, expression of the N-terminal fragment of MAPKK which encompasses the nuclear export signal sequence and the MAPK-binding site blocked both the serum-induced S phase entry of quiescent cells and the oncogenic Ras-induced morphological changes. All these results demonstrate that MAPKK is one of key molecules involved in the control of both cell morphology and cell proliferation and suggest an important role for the N-terminal region of MAPKK in the regulation of the MAPK signaling.

Cells use a limited number of intracellular signaling pathways for a variety of cellular responses. One of these pathways is the classical mitogen-activated protein kinase (MAPK) cascade that is involved in the regulation of cell proliferation, cell differentiation, and embryonic early development. In response to extracellular stimuli MAPK kinase kinase (MAPKK, also known as MEK), a direct activator for MAPK/extracellular signal-regulated kinase, becomes activated and then activates MAPK in the cytoplasm (1–3). The activated MAPK is translocated into the nucleus (4–6) where it phosphorylates several nuclear targets such as Elk-1 (7, 8). This signaling event is thought to stimulate the induction of an immediate early gene fos (9), which may, with some other transcription factors, activate many late genes required for the S phase entry of quiescent fibroblastic cells. Several experiments revealed a requirement of MAPK for serum-induced cell growth (10, 11). Thus, the MAPK cascade is important for the initiation of cell proliferation.

Ras is an upstream regulator of the MAPK cascade. Oncogenic Ras induces malignant transformation of fibroblastic cells, and as earlier cell responses, it causes both the S phase entry and the morphological changes (12, 13). A dominant-negative form of MAPKK blocks oncogenic Ras-induced cell transformation (14), and overexpression of MAPK phosphatase, MKP1/CL100, inhibits Ras-induced S phase entry of quiescent cells (15). These results suggest that the activation of the MAPK cascade is essential for the oncogenic Ras-initiated cell proliferation and transformation. Thus, the classical MAPK cascade has been thought to function primarily as a mediator of mitogenic signals through gene expression. On the other hand, little has been known about its role in the morphological changes induced by Ras.

Constitutively active mutants of MAPKK are successfully generated by substituting acidic amino acids for two serine residues in the activation phosphorylation sites (16–20). These mutants are shown to be able to induce malignant cell transformation of fibroblasts (14, 20), differentiation of PC12 cells (14), oocyte maturation (21, 22), and mesoderm induction in Xenopus (23–25). However, there have been no reports demonstrating the ability of constitutively active MAPKK to induce the S phase entry of quiescent cells or early morphological changes.

We previously showed that a leucine-rich nuclear export signal (NES) (residues 33–44) in the N-terminal region of MAPKK directs cytoplasmic localization of MAPKK (26). Disruption of the NES by substituting alanines for essential leucines dramatically enhanced the ability of constitutively active MAPKK to induce malignant cell transformation (27), suggesting a potential role for the NES of MAPKK to suppress abnormal cellular responses. Moreover, it has been shown that MAPKK binds to the N-terminal region of MAPKK (28). Through this binding, MAPKK appears to be retained to the cytoplasm (28). Extracellular stimuli may induce dissociation of this MAPKK-MAPK complex, and MAPKK is then translocated to the nucleus (28). Thus, MAPKK may function as a cytoplasmic anchor for MAPK.

In this study, we show evidence that the MAPK cascade is involved in the control of cell morphology and is sufficient for initiation of cell proliferation. We then demonstrate dominant-negative effects of the N-terminal region of MAPKK on the MAPK cascade signaling.

**EXPERIMENTAL PROCEDURES**

DNA Constructions—pShHa-MAPKK and pShHa-LA-SDSE MAPKK were produced as described (27). pShHa-SASA MAPKK was generated from an EcoRI fragment of Xenopus MAPKK cDNA (29) subcloned into M13mp18 by the methods of Kunkel et al. (30) using a mutant primer 5′-GGGCAACTCTAGACGCGTCCATCGGAAAATGCT-
TTTGGGCAAGATCC-3' as described (27). The open reading frame of SASA MAPKK was amplified by polymerase chain reaction with a 5' primer, 5'-ACTCAGATCTTACATGCTAAAAGAAG-3', and a 3' primer, 5'-GCGGGATCCTGGGATCCCGCCGAG-3', which produce BglII sites at both ends of SASA MAPKK. The BglII fragment of SASA MAPKK was cloned into pSRoHA1, yielding pSRoHA-SASA MAPKK. An N-terminal region (residues 1-60) of wild-type MAPKK and that of LA MAPKK (26) were amplified by polymerase chain reaction with a 5' primer, 5'-CCGGGATCCTCGGTATAAAAGAAGCGGTCAT-3', and a 3' primer, 5'-GCGGGATTCGCTTCGTTCTCGGTATAAAAGAAGCGGTCAT-3', which produce a BamHI site at the 5'-terminal and an EcoRI site at the 3'-terminal. Each BamHI-EcoRI fragment was cloned into pSRoHA1, yielding pSRoHA-MAPKK(1-60) and pSRoHA-LA-MAPKK(1-60), respectively. pSRo-Ras° was kindly given by Dr. S. Hattori. The BglII fragment of LA-MAPKK was cloned into pET-16b (Novagen) to produce pET-16b-LA-SDSE MAPKK (His-LA-SDSE MAPKK). The open reading frame of Xenopus MAPKK was cloned into pcDL-SRα57 to obtain pSRo-MAKP.

Preparation of Recombinant Proteins—Histidine-tagged Xenopus MAPKK (His-MAPKK) and histidine-tagged Xenopus LA-SDSE MAPKK (His-LA-SDSE MAPKK) were expressed in Escherichia coli strain BL21 (DE3) pLYS S pT-Trx by incubating with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 5 h at 25 °C and purified as described (17). His-MAPKK (2 mg/ml) and His-LA-SDSE MAPKK (2 mg/ml) were dialyzed against the injection buffer (20 mM K-Hepes, pH 7.4, 120 mM KCl) before injection.

Cells—Swiss 3T3 cells and MDCK cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics (100 units/ml penicillin and 0.2 mg/ml kanamycin). For microinjection, cells were plated on glass coverslips or CELLocate coverslips (Eppendorf, Inc.), and grown as above to confluence. For Fos induction and BrdUrd incorporation assays, Swiss 3T3 cells were plated as above, grown to confluence, and then left overnight in serum-free medium to ensure that all the cells had entered the quiescent state.

Fig. 2. Effect of anti-Ras neutralizing antibody on MAPKK-induced morphological changes. A, Y13-259 blocks serum-induced S phase entry of quiescent Swiss 3T3 cells. A neutralizing antibody against Ras (Y13-259) (10 mg/ml) or control rat IgG (10 mg/ml) was injected into the cytoplasm of serum-starved confluent Swiss 3T3 cells. 1 h after injection, cells were stimulated with 20% FCS, cultured for 30 h in the presence of BrdUrd, and then stained with antibody to BrdUrd followed by an FITC-labeled secondary antibody. Injected IgG was visualized by staining with an FITC-labeled goat anti-rat antibody. Arrowheads indicate IgG-injected cells. B, Y13-259 does not block MAPKK-induced morphological changes. Y13-259 (10 mg/ml) was pre-injected into the cytoplasm of several cells inside the marked area of coverslips. 30 min after injection, pSRoHA-LA-SDSE MAPKK (200 μg/ml) was injected into the nuclei of the cells in the same area. After 18 h, cells were stained with antibody to HA tag (MAPKK). Injected Ras antibody was visualized with an FITC-labeled goat anti-rat antibody (Ras antibody). In phase contrast images, black arrowheads indicate the cells injected with both the antibody and the MAPKK plasmid, whereas white arrowheads indicate the cells injected with only MAPKK. Surrounding un.injected cells also showed slight signs of morphological changes. At least 50 cells were injected with Y13-259, and no cells showed signs of reverted morphologies. Experiments were repeated 3 times and gave similar results.

Fig. 1. Morphological changes of fibroblastic and epithelial cells induced by LA-SDSE MAPKK. A, morphological changes and disruption of focal adhesions in Swiss 3T3 cells expressing LA-SDSE MAPKK. Swiss 3T3 cells were grown on the coverslips. pSRoHA-MAPKK (WT, 350 μg/ml) or pSRoHA-LA-SDSE MAPKK (LA-SDSE, 350 μg/ml) was injected into the nuclei of cells. After 18 h, the cells were stained with antibody to HA (12CA5) or antibody to vinculin, followed by FITC- or fluorescein isothiocyanate-conjugated secondary antibodies. The rhodamine and phase contrast images of one representative field for each sample are shown (upper four panels). Vinculin-staining images of the plasmid-injected cells (lower two panels) were 3 times as magnified as upper four panels. B, effect of LA-SDSE MAPKK expression on MDCK cells. pSRoHA-MAPKK (WT, 350 μg/ml) or pSRoHA-LA-SDSE MAPKK (LA-SDSE, 350 μg/ml) was injected into the nuclei of MDCK cells in a single colony. After 18 h, cells were stained with 12CA5 followed by an fluorescein isothiocyanate-conjugated secondary antibody. Fluorescein and phase images of each representative field are followed by an fluorescein isothiocyanate-conjugated secondary antibody. Fluorescein and phase images of each representative field are shown. Cytoplasmic localization of WT MAPKK is not so clear in MDCK cells (lower two panels) as compared with that for WT MAPKK because of the more rounded cell morphologies. Staining intensity for LA-SDSE MAPKK is a little bit stronger than that for WT MAPKK, indicating that LA-SDSE MAPKK is a little bit stronger than WT MAPKK.

Arrowheads indicate the cells injected with both the antibody and the MAPKK plasmid, whereas white arrowheads indicate the cells injected with only MAPKK. Surrounding un injected cells also showed slight signs of morphological changes. At least 50 cells were injected with Y13-259, and no cells showed signs of reverted morphologies. Experiments were repeated 3 times and gave similar results.
Microinjection—Microinjection was performed with an IM-188 apparatus (Narishige) as described previously (26). An anti-Ras mAb Y13-259 (10 mg/ml) and control rat IgG (10 mg/ml) were dialyzed against the injection buffer (see “Preparation of Recombinant Proteins”) before injection. For BrdUrd incorporation assay, BrdUrd (0.5 mg/ml) and insulin (1 μg/ml) were added to the media and incubated for 30 h. All the cells inside the specified circle of each marked coverslip were injected. Cells were stained for BrdUrd incorporation into the nuclei (BrdU). Cell nuclei were also visualized with DAPI. The purity of each protein is more than 90%. The activity of recombinant His-LA-SDSE MAPKK protein to phosphorylate kinase-negative Xenopus MAPK was about 45 times as high as that of WT MAPKK protein (data not shown). More than 10 circles were injected for one experiment. Experiments were repeated 3 times with similar results.

Cell Staining—Cells were fixed and permeabilized as described previously (26). To detect BrdUrd-positive cells, cells were incubated for 40 min at 37 °C with anti-BrdUrd mAb (5 μg/ml, Becton Dickinson), DNase I (0.5 mg/ml), and 0.1% bovine serum albumin followed by an RITC-conjugated goat anti-mouse antibody (Cappel) and DAPI (0.1 mg/ml). Hemagglutinin (HA)-tagged MAPKK proteins were detected with anti-HA mAb (12CA5). Focal adhesions were visualized with antivinculin mAb (VIN-11–5). Anti-Ras mAb (Y13-259) was a kind gift from Dr. S. Hattori. Injected Ras antibody and control rat IgG were detected with RITC-conjugated anti-rat antibody (Cappel). Anti-Fos antibody was purchased from Upstate Biotechnology Inc. An anti-Xenopus MAPK antibody was produced in rabbit by using bacterially expressed recombinant His-tagged MAPK as an antigen. Fluorescence and phase contrast images were observed in an Axiophot (Zeiss) with a 63× plan-Neofl. (1.25 NA) objective.

RESULTS

Activation of MAPKK Induces Early Morphological Changes Independent of Cellular Ras Function—Although the cell trans-
formation by constitutively active MAPKK is often accompanied by morphological changes of the cell, the effect of the MAPKK activation on early (≤ 24 h) morphological changes has been poorly understood. To examine the effect of activation of MAPKK on cell morphology in fibroblasts, we injected the nuclei of subconfluent Swiss 3T3 cells with a mammalian expression vector encoding an NES-disrupted, constitutively active MAPKK, LA-SDSE MAPKK in which leucines in NES are replaced by alanines (35), was co-expressed with oncogenic Ras. Expression of SASA MAPKK, but not that of WT MAPKK, sometimes showed slight morphological changes. The altered morphology of constitutively active MAPKK-expressing cells might not be a direct consequence of the activation of the MAPK cascade but rather result from some autocrine mechanism that leads to the activation of Ras which might induce morphological responses independent of the MAPK cascade (14). To address this, we investigated the effect of a neutralizing antibody against Ras, Y13-259, on the morphological changes induced by LA-SDSE MAPKK. The monoclonal antibody Y13-259 has been shown to block cellular H-, K-, and N-Ras function (31, 32), and in agreement with a previous report with NIH 3T3 cells (31), injection of Y13-259 into the cytoplasm blocked serum-induced S phase entry of quiescent Swiss 3T3 cells (Fig. 2A, Y13-259). Blocking efficiency was quite high when the entry of S phase was determined by incorporation of bromodeoxyuridine (BrdUrd) into the nucleus; only 6 out of 100 injected cells incorporated BrdUrd in the first experiment and 8 out of 100 in the second experiment. Control rat IgG did not block BrdUrd incorporation at all (Fig. 2A, cont. IgG). These results confirm that Y13-259 is a strong and specific neutralizing antibody. Y13-259 was pre-injected into the cytoplasm of Swiss 3T3 cells inside the marked area of coverslips, and after 30 min the plasmid DNA encoding LA-SDSE MAPKK was injected into the nucleus of these cells. As shown in Fig. 2B, morphological changes, typical of those induced by LA-SDSE MAPKK, occurred even in the Y13-259-injected cells. The cells became rounded and shrunk, appeared refractile, and had some protrusions and processes. The antibody-injected cells were indistinguishable from un.injected cells. These results suggest that constitutively active MAPKK-induced morphological changes do not require Ras function. Consistent with this, expression of a dominant-negative form of Ras did not inhibit LA-SDSE MAPKK-induced morphological changes (data not shown). Therefore, it is suggested that morphological changes are primarily downstream events from MAPKK, although some effect through possible autocrine mechanism cannot be ruled out because cells surrounding the active MAPKK-expressing cells sometimes showed slight morphological changes.

Then we tested whether oncogenic Ras-induced early morphological changes are mediated through a function of MAPKK. When an expression vector encoding RasV12 was injected into the nuclei of Swiss 3T3 cells, the cells became rounded, shrunk, and refractile, and numerous pinocytotic vacuoles appeared (33) (Fig. 3A, –). An inhibitor of MAPKK activation, PD098059 (34), significantly inhibited these phenotypes (Fig. 3A, PD098059), whereas Me2SO (a solvent for PD098059) had no effect (Fig. 3A, DMSO). To confirm further a requirement of MAPKK for induction of these phenotypes by Ras, WT MAPKK, or SASA MAPKK (with an HA tag), a dominant-negative form of MAPKK in which two activating serines are replaced by alanines (35), was co-expressed with oncogenic Ras. Expression of SASA MAPKK, but not that of WT MAPKK,
for each sample. At least 100 cells were injected BrdUrd-positive cells in the injected cells. The staining intensity for HA tag confirmed the same level of expression of WT and SASA MAPKK. These results, together with the observation that inhibition of Ras did not interfere with the ability of active MAPKK to induce morphological changes (Fig. 2B), suggest a linkage between the classical MAPK cascade and the control of cell morphology.

Activation of MAPKK Is Sufficient for Initiation of Cell Proliferation—Although previous studies suggested the importance of the MAPK cascade in the initiation of cell proliferation, it has not been determined whether activation of the MAPK cascade alone is sufficient for induction of an immediate early gene fos and the S phase entry of quiescent cells. To test this, we first injected the MAPKK plasmids into the nuclei of quiescent Swiss 3T3 cells, and induction of endogenous Fos was examined. Expression of LA-SDSE MAPKK, but not that of WT MAPKK, induced endogenous Fos, as revealed by the appearance of bright nuclear staining with anti-Fos antibody (Fig. 4A). This level of Fos induction was roughly the same as the level of Fos that was induced by stimulation with 20% fetal calf serum, as judged by the staining intensity (data not shown). Then, to examine the S phase entry, we produced bacterially expressed WT and LA-SDSE MAPKK proteins, and we injected each of them into the cytoplasm of all the quiescent Swiss 3T3 cells and induction of endogenous Fos was examined. Expression of LA-SDSE MAPKK, but not that of WT MAPKK, induced endogenous Fos, as revealed by the appearance of bright nuclear staining with anti-Fos antibody (Fig. 4A). This level of Fos induction was roughly the same as the level of Fos that was induced by stimulation with 20% fetal calf serum, as judged by the staining intensity (data not shown). Then, to examine the S phase entry, we produced bacterially expressed WT and LA-SDSE MAPKK proteins, and we injected each of them into the cytoplasm of all the quiescent Swiss 3T3 cells inside the specified circle on the marked coverslips. After injection, cells were incubated for 30 h in the presence of insulin (36) and BrdUrd. Whereas none of WT MAPKK-injected cells incorporated BrdUrd, approximately 40% of LA-SDSE MAPKK-injected cells incorporated BrdUrd (Fig. 4B, BrdUrd). Therefore, activation of the MAPK cascade may be sufficient for the S phase entry of quiescent cells, as well as the induction of Fos.

Expression of the N-terminal Region of MAPKK Causes Dominant-negative Effects—In our previous report we proposed a potential role of NES of MAPKK to prevent malignant cell transformation (27). To test whether the NES-containing region has regulatory roles in the serum-induced G0/S transition and the Ras-induced morphological changes, we produced expression vectors encoding an HA-tagged N-terminal fragment of WT or NES-disrupted (LA) MAPKK (1–60 amino acids) (Fig. 5A). This region contains both the MAPK-binding site (residues 1–32) and the NES sequence (residues 33–44) (28). We expressed each of these fragments together with MAPK in Swiss 3T3 cells and determined their intracellular localization. The WT fragment was localized in the cytoplasm, whereas the LA fragment was present in both the nucleus and the cytoplasm. MAPKK was localized in the cytoplasm when co-expressed with the WT fragment, whereas it was present in both the nucleus and the cytoplasm when expressed with the LA fragment (Fig. 5B).

Then we expressed these fragments in quiescent Swiss 3T3 cells, and we examined the effect on the serum-induced S phase entry of cells. Expression of an empty vector did not affect 20% fetal calf serum-induced BrdUrd incorporation into the nucleus (Fig. 6A). Expression of the WT fragment, however, blocked the serum-induced S phase entry, whereas expression of the LA fragment did not significantly inhibit the BrdUrd incorporation (Fig. 6A). Fig. 7B represents a quantification of the result; 78% of vector-injected cells entered the S phase under the conditions, whereas none of the WT fragment-expressing cells entered the S phase, and 54% of the LA fragment-expressing cells incorporated BrdUrd.

Finally, we examined the effect of these fragments on the oncogenic Ras-induced morphological changes. Each of these fragments was co-expressed with RasV12 in subconfluent Swiss 3T3 cells. Expression of the WT fragment inhibited the Ras-induced morphological changes; the cells were flattened and had few pinocytotic vacuoles and few protrusions (Fig. 7, +
blocks oncogenic Ras-induced early morphological changes. pSRα-RasV12 (200 μg/ml) was co-injected with pSRαHA-MAPKK-(1–60) (+ WT) or pSRαHA-LA-MAPKK-(1–60) (+ LA) (300 μg/ml for each) into the nuclei of Swiss 3T3 cells. After 18 h, cells were fixed and stained as in Fig. 3B. At least 100 cells were injected, and a representative field for each sample is shown. Experiments are repeated 3 times with similar results.

### Discussion

Although it was previously shown that the MAPK pathway is involved in a variety of cellular responses, the relationship between the MAPK cascade and the cell morphology remained undefined. In addition, it was also unclear whether the MAPK pathway was actually able to induce the S phase entry of quiescent cells. By using a constitutively active mutant of MAPKK (LA-SDSE MAPKK) whose kinase activity is the highest in the recombinant MAPKK mutants but still lower than that of normally activated, dually phosphorylated MAPKK (27), we have here shown a link between the MAPK cascade and the control of cell morphology, and we have also shown the ability of the MAPK cascade to induce the expression of Fos and the S phase entry.

A number of experiments suggested that Ras induces morphological changes and gene expression through different effector proteins (37, 38). The Raf/MAPK cascade, one of the downstream components of Ras, was thought to function to transmit mitogenic signals to the nucleus to induce gene expression and not to participate in the regulation of cell morphology or actin reorganization (14, 38). The present results demonstrate that active MAPKK is able to induce dramatic changes in cell morphology which are accompanied by disruption of both actin stress fibers and focal adhesions and that inhibition of Ras function does not suppress the MAPKK-induced morphological changes. Moreover, we observed that inhibition of MAPKK or expression of the N-terminal portion of MAPKK suppresses oncogenic Ras-induced morphological changes. These results therefore suggest that the Ras-dependent morphological changes are at least partly mediated by the MAPKK/MAPK cascade. Downstream events or target molecules of MAPK which are involved in morphological changes remain to be elucidated. It has been reported that activation of Raf suppresses integrin activation probably through MAPK activation (39). It is possible that the MAPK cascade affects cytoskeletal network and cell adhesion machinery through phosphorylation of cytoplasmic target proteins.

Finally we have shown dominant-negative effects of the N-terminal fragment of MAPKK on the MAPK signaling. One of possible molecular mechanisms for these effects is that the fragment with the normal NES retains endogenous MAPK in the cytoplasm through the MAPK-binding site and thus suppresses nuclear import of MAPK in response to upstream stimuli. The other is that the fragment inhibits the complex formation between MAPK and MAPKK. Recently it has been reported that a bacterial toxin which causes anthrax of animals inhibits the MAPK signaling by cleaving an N-terminal region of MAPKK (1–7 amino acids of human MAPKK1) (41). Since this region may contribute to the MAPK binding (28), the cleavage may prevent the complex formation between MAPKK and MAPK. Thus, it is likely that the complex formation between MAPKK and MAPK contributes to an increase in efficiency of the MAPKK-induced MAPK activation in normal signal transmission.

In conclusion, our results here suggest that MAPKK is a key molecule in the Ras pathway, controlling both cell morphology and cell proliferation, and demonstrate an important regulatory role of the N-terminal region of MAPKK for the MAPK signaling. The results might also explain an underlying mechanism through which constitutive activation of the MAPK cascade alone can lead to cell transformation with typical morphological alterations.

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