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Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase

Makoto Fukuda, Yukiko Gotoh and Eisuke Nishida[1,2]

Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

[1]Present address: Department of Biophysics, Graduate School of Science, Kyoto University, Kitashirakawa-Oiwake, Sakyo-ku, Kyoto 606-01, Japan

[2]Corresponding author

The mitogen-activated protein kinase (MAPK) cascade consisting of MAPK and its direct activator, MAPK kinase (MAPKK), is essential for signaling of various extracellular stimuli to the nucleus. Upon stimulation, MAPK is translocated to the nucleus, whereas MAPKK stays in the cytoplasm. It has been shown recently that the cytoplasmic localization of MAPKK is determined by its nuclear export signal (NES) in the near N-terminal region (residues 33–44). However, the mechanism determining the subcellular distribution of MAPK has been poorly understood. Here, we show that introduction of v-Ras, active STE11 or constitutively active MAPKK can induce nuclear translocation of MAPK in mammalian cultured cells. Furthermore, we show evidence suggesting that MAPK is localized to the cytoplasm through its specific association with MAPKK and that nuclear accumulation of MAPK is accompanied by dissociation of a complex between MAPK and MAPKK following activation of the MAPK pathway. We have identified the MAPK-binding site of MAPKK as its N-terminal residues 1–32. Moreover, a peptide encompassing the MAPK-binding site and the NES sequence of MAPKK has been shown to be sufficient to retain MAPK to the cytoplasm. These findings reveal the molecular basis regulating subcellular distribution of MAPK, and identify a novel function of MAPKK as a cytoplasmic anchoring protein for MAPK.

Keywords: MAP kinase/MAP kinase kinase/nuclear export signal/nuclear translocation/signal transduction

Introduction

Mitogen-activated protein kinase (MAPK) is activated by dual phosphorylation catalyzed by MAPK kinase (MAPKK), and MAPKK is activated by serine phosphorylation catalyzed by MAPKK kinase (MAPKKK) (Sturgill and Wu, 1991; Ahn *et al.*, 1992; Thomas, 1992; Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Marshall, 1994; Cobb and Goldsmith, 1995). This MAPK cascade plays a central role in a wide variety of signaling pathways (Haccard *et al.*, 1993; Pages *et al.*, 1993; Cowley *et al.*, 1994; Kosako *et al.*, 1994; Mansour *et al.*, 1994; Gotoh *et al.*, 1995; LaBonne *et al.*, 1995; Umbhauer *et al.*, 1995). In response to extracellular stimuli, the terminal component of three kinases, MAPK, is translocated from the cytoplasm to the nucleus (Chen *et al.*, 1992; Sanghera *et al.*, 1992; Traverse *et al.*, 1992, 1994; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993; Dikic *et al.*, 1994), where activated MAPK phosphorylates and regulates nuclear proteins including transcription factors. The prolonged activation of MAPK and its nuclear translocation are suggested to be important for neural differentiation of PC12 cells (Traverse *et al.*, 1992, 1994; Dikic *et al.*, 1994) and initiation of DNA synthesis in fibroblasts (Lenormand *et al.*, 1993). The cell staining with anti-active MAPK antibody has shown that upon mitogenic stimulation of fibroblastic cells MAPK is activated primarily in the cytoplasm, and the activated MAPK appears to move to the nucleus (our unpublished data). These observations imply that nuclear translocation of MAPK is a crucial step in signal transduction, and phosphorylation and/or activation of MAPK may be important for its nuclear translocation. However, the mechanism controlling this step has not been elucidated.

In contrast to MAPK, MAPKK stays in the cytoplasm to transmit the signal from the plasma membrane to MAPK in the cytoplasm (Lenormand *et al.*, 1993; Zheng and Guan, 1994; Moriguchi *et al.*, 1995). We have shown recently that this cytoplasmic localization of MAPKK is determined by its nuclear export signal (NES) sequence in the near N-terminal region (residues 33–44) (Fukuda *et al.*, 1996). The first identification of NES (Bogerd *et al.*, 1995; Fischer *et al.*, 1995; Stuzt *et al.*, 1995; Wen *et al.*, 1995) was carried out recently in studies characterizing two specific proteins, human immunodeficiency virus-1 (HIV-1)-coded Rev protein and inhibitor (PKI) of cAMP-dependent protein kinase, that rapidly shuttle between the nucleus and cytoplasm. The NES sequences in Rev and PKI are both rich in hydrophobic residues which are critical for nuclear export activity (Bogerd *et al.*, 1995; Fischer *et al.*, 1995; Stuzt *et al.*, 1995; Wen *et al.*, 1995). The NES sequence of MAPKK is rich in leucine residues, which were also revealed to be crucial for its NES activity (Fukuda *et al.*, 1996). Thus, when the NES activity was disrupted by replacement of the critical leucines by alanines, MAPKK became present both in the nucleus and in the cytoplasm, unlike wild-type MAPKK which was present exclusively in the cytoplasm and excluded from the nucleus (Fukuda *et al.*, 1996).

Since nuclear translocation of MAPK is induced to occur by those stimuli that induce activation of the MAPK cascade, and as activation of MAPK by MAPKK occurs in the cytoplasm, it is reasonable to assume the existence of some mechanism linking activation of the MAPK cascade to MAPK nuclear translocation. Two previous observations (Gonzalez *et al.*, 1993; Lenormand *et al.*,

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1993) seem important in understanding the mechanism determining the subcellular distribution of MAPK. One is that a kinase-deficient mutant or non-phosphorylated mutants of MAPK, like wild-type MAPK, can be translocated to the nucleus in response to serum stimulation. The other is that expression of excess amounts of MAPK results in its nuclear accumulation even in the absence of the stimulation. Any proposed mechanism for nuclear translocation of MAPK should take these observations into consideration. Here we first show that activation of MAPKK is sufficient for inducing nuclear translocation of MAPK. Then, we show evidence that MAPK is located in the cytoplasm in unstimulated cells through its specific association with MAPKK, and that nuclear accumulation of MAPK is accompanied by dissociation of a complex between MAPK and MAPKK folowing activation of the MAPK pathway. Furthermore, we have identified the region of MAPKK responsible for its ability to retain MAPK in the cytoplasm. Thus, this study reveals a novel function of MAPKK as a cytoplasmic anchoring protein for MAPK.

Results and discussion

Nuclear translocation of MAPK is induced by activation of the MAPK pathway

When bacterially expressed *Xenopus* MAPK and bovine serum albumin (BSA) were injected into the cytoplasm of quiescent rat fibroblastic 3Y1 cells and the cells were exposed to 10% fetal calf serum (FCS) plus 30 nM epidermal growth factor (EGF), the injected MAPK crossed the nuclear envelope of injected cells and was localized in the nucleus (Figure 1A). In the absence of serum/EGF stimulation, the injected MAPK was predominantly in the cytoplasm (Figure 1A). In contrast, injected BSA was excluded from the nucleus even after the stimulation (Figure 1A). To examine whether or not activation of the MAPK cascade is capable of inducing nuclear translocation of MAPK, we first used v-Ras p21 as an activator for the MAPK cascade. Co-injection of bacterially expressed v-Ras with MAPK into the cytoplasm induced nuclear translocation of the injected MAPK even in the absence of serum/EGF stimulation (Figure 1A). Thus, activation of Ras may be sufficient to induce nuclear translocation of MAPK. Then, we used an N-terminal truncated STE11 (ΔN-STE11), a constitutively active form of yeast MAPKKK (Errede and Levin, 1993), to induce activation of MAPKK. ΔN-STE11 was expressed in bacteria as a GST fusion protein and purified. This recombinant ΔN-STE11 protein (130 μg/ml) induced almost full activation of both MAPKK and MAPK within 30 min in a cell-free system consisting of *Xenopus* immature oocyte extracts and the ATP-regenerating system at 25°C (K.Takenaka *et al.*, unpublished data). Cytoplasmic injection of bacterially expressed ΔN-STE11 with MAPK or BSA induced nuclear translocation of MAPK, but not BSA, in a time-dependent manner (Figure 1B).

As the MAPKK/MAPK cascade is the major downstream signaling pathway common to both v-Ras and ΔN-STE11 (Blenis, 1993; Davis, 1993; Errede and Levin, 1993; Nishida anf Gotoh, 1993; Marshall, 1994), we next tested whether active MAPKK could induce the nuclear translocation of MAPK. We constructed a plasmid harboring a constitutively active mutant of *Xenopus* MAPKK (SDSE-MAPKK) (Mansour *et al.*, 1994) and co-expressed it with a low level of hemagglutinin-tagged *Xenopus*

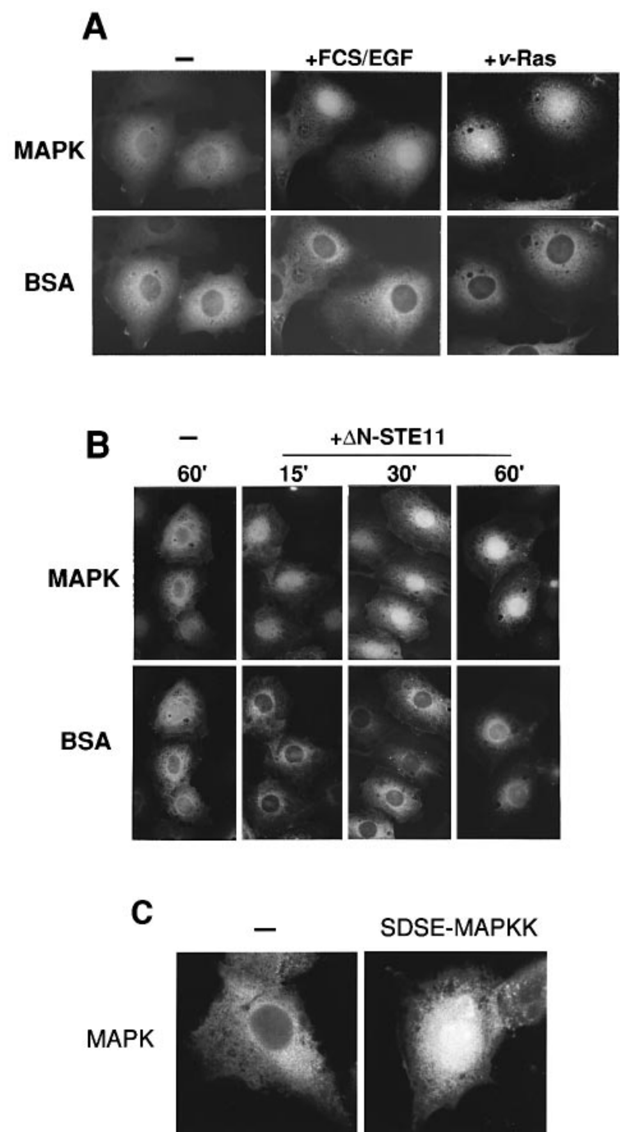

Fig. 1. Nuclear translocation of MAPK by v-Ras (A), active MAPKK-K (B) and constitutively active MAPKK (C). (**A**) The cytoplasm of quiescent rat fibroblastic 3Y1 cells was injected with recombinant *Xenopus* MAPK (0.8 mg/ml) and tetramethyl rhodamine isothiocyanate-labeled BSA (TRITC-BSA; 1.0 mg/ml), and the cells were left in serum-free medium (–) or exposed to 10% FCS and 30 nM EGF (+FCS/EGF). In another series of experiments, recombinant *Xenopus* MAPK, TRITC-BSA and v-Ha-Ras (8.0 mg/ml) were injected into the cytoplasm of quiescent 3Y1 cells (+v-Ras), and the cells were cultured in serum-free medium. Cells were then fixed at 1 h after injection, and stained for the injected *Xenopus* MAPK with rabbit antiserum against *Xenopus* MAPK followed by FITC-conjugated secondary antibody. The location of the injected BSA in the same cells was also determined. (**B**) A mixture of recombinant MAPK and TRITC-labeled BSA with (+ΔN-STE11) or without (–) bacterially expressed ΔN-STE11 (1.0 mg/ml) was injected into the cytoplasm of quiescent 3Y1 cells. ΔN-STE11 is an active form of STE11, a yeast MAPKKK. The subcellular localization of two proteins, the injected MAPK and BSA, was determined at the indicated times. (**C**) Rat 3Y1 cells were injected with a plasmid harboring HA-tagged MAPK (50 μg/ml) with or without a plasmid harboring a constitutively active *Xenopus* MAPKK (SDSE-MAPKK, 100 μg/ml). Cells were stained with anti-MAPK antibody at 6 h after injection to determine the location of HA-MAPK.

MAPK (HA-MAPK). The kinase activity of SDSE-MAPKK was ~30-fold higher than that of WT-MAPKK (data not shown). The cell staining with anti-MAPK antibody or anti-HA antibody (data not shown) showed clearly that expression of SDSE-MAPKK induced nuclear accumulation of HA-MAPK (Figure 1C). These results suggest that activation of MAPKK is sufficient for the nuclear translocation of MAPK.

Overexpression of MAPK results in its nuclear accumulation, which is not prevented by WGA

Lenormand *et al.* (1993) reported previously that MAPK became localized in the nucleus even in the absence of stimulation when it was expressed stably at high levels. To examine in detail the effect of increasing concentrations of MAPK on its subcellular distribution, we injected a fixed amount of a plasmid harboring HA-MAPK together with increasing amounts of a non-tagged *Xenopus* MAPK (MAPK) plasmid into the nuclei of rat 3Y1 cells, and determined the subcellular location of HA-MAPK by staining with anti-HA antibody. The expression of HA-MAPK alone resulted in its diffuse distribution throughout the cell, while co-expression of MAPK at high levels induced the marked nuclear accumulation of HA-MAPK in a dose-dependent manner (Figure 2A). We also observed that injection of a large amount of bacterially expressed recombinant MAPK protein into the cytoplasm of unstimulated cells resulted in its nuclear accumulation (data not shown and see Figure 4A). These results may imply that MAPK is somehow retained in the cytoplasm in unstimulated cells and that this putative cytoplasmic retention of MAPK is a saturable process.

Interestingly, the nuclear accumulation of MAPK was not inhibited by wheat germ agglutinin (WGA), while nuclear translocation of nuclear localization signal (NLS)-conjugated BSA was completely inhibited by WGA (Finlay *et al.*, 1987; Yoneda *et al.*, 1987) (Figure 2B). This result suggests that nuclear accumulation of MAPK is not an NLS-dependent process. In fact, there are no obvious NLSs in the sequence of MAPK.

Association of MAPKK with MAPK in the cytoplasm

We then examined the effect of co-expression of MAPKK on the MAPK location. Remarkably, co-expression of MAPKK in unstimulated cells caused a drastic change in the MAPK distribution; MAPK became restricted to the cytoplasm and excluded from the nucleus (Figure 2C, WT-KK/MAPK). This unexpected finding suggests that MAPKK may function to retain MAPK in the cytoplasm. To examine this idea, we tested the possibility that MAPK and MAPKK could form a complex in cells. HA-MAPKK and MAPK were co-expressed in COS-7 cells, and cell lysates were subjected to immunoprecipitation with anti-HA antibody to precipitate MAPKK. The immunoblotting of the precipitate with anti-MAPK antibody revealed a clear co-precipitation of MAPK with MAPKK (Figure 3A, lane 4). HA-MAPKK was also co-immunoprecipitated with MAPK when MAPK was immunoprecipitated with anti-MAPK antibody (data not shown). When cell lysates obtained from cells expressing only MAPK were subjected to immunoprecipitation with anti-HA antibody, no MAPK

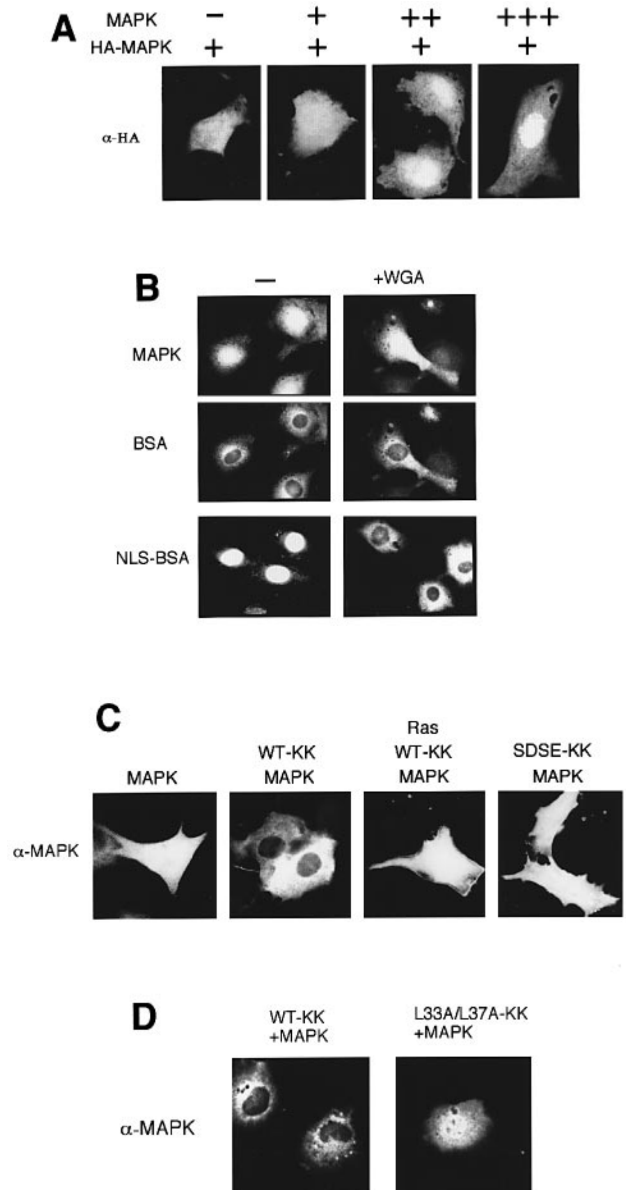

Fig. 2. (**A**) Overexpression of MAPK results in its nuclear accumulation. Rat 3Y1 cells were injected with a plasmid harboring HA-tagged MAPK (HA-MAPK; 80 μg/ml) together with a plasmid harboring non-tagged MAPK (MAPK; –, 0 μg/ml; +, 40 μg/ml; ++, 80 μg/ml; +++, 160 μg/ml). Cells were stained with an antibody against HA (αHA; 12CA5) at 14 h after injection to determine the subcellular distribution of HA-MAPK. (**B**) The nuclear accumulation of MAPK is not inhibited by WGA. Recombinant *Xenopus* MAPK protein (MAPK; 2 mg/ml) plus TRITC-labeled BSA (BSA; 1 mg/ml) or FITC-labeled nuclear localization signal (NLS) peptide-conjugated BSA (NLS–BSA; 0.3 mg/ml) with or without 1 mg/ml WGA was injected into the cytoplasm of cells, and their subcellular distribution was determined after 1 h. (**C**) The nuclei of rat 3Y1 cells were injected with a MAPK plasmid (MAPK; 100 μg/ml) together with a wild-type MAPKK plasmid (WT-KK; 150 μg/ml), a WT-KK plasmid plus a Ras^{val12} plasmid (Ras; 100 μg/ml) or a constitutively active MAPKK plasmid (SDSE-KK; 150 μg/ml). The subcellular distribution of MAPK was determined 14 h after injection by anti-*Xenopus* MAPK antibody. In this series of experiments, essentially the same results were obtained when these plasmids were transfected in COS-7 cells (see A). (**D**) COS-7 cells were transiently co-transfected with the MAPK plasmid and the WT-MAPKK plasmid (left) or the NES-disrupted MAPKK plasmid (L33A/L37A-KK, right) as described in Materials and methods. Cells were then stained with anti-*Xenopus* MAPK antibody at 16 h after transfection to determine the subcellular distribution of MAPK.

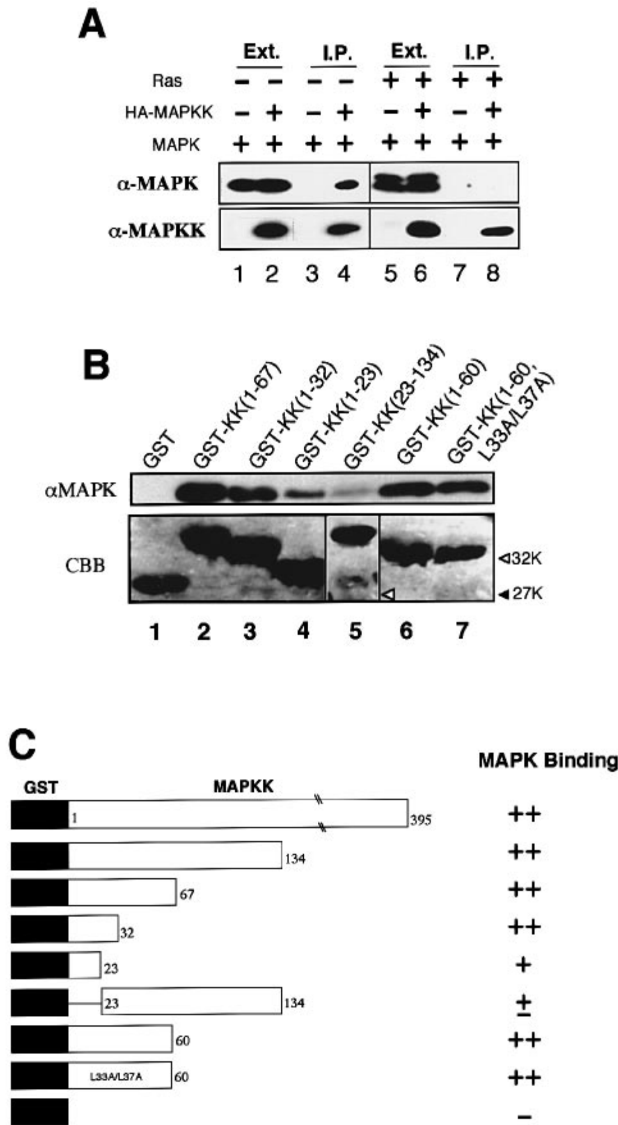

Fig. 3. (**A**) Association of MAPK with MAPKK in intact cells. COS-7 cells were transfected with MAPK alone (lanes 1 and 3), with MAPK and HA-MAPKK (lanes 2 and 4), with MAPK and Ras^{val12} (lanes 5 and 7) or with MAPK and HA-MAPKK plus Ras^{val12} (lanes 6 and 8). Lysates from transfected cells were run directly on an SDS gel (Ext.) or subjected to immunoprecipitation with anti-HA monoclonal antibody (I.P.). After SDS–PAGE, the samples were immunoblotted with anti-MAPK antibody or anti-MAPKK antibody. (**B**) MAPK interacts with the N-terminal region of MAPKK *in vitro*. Bacterially expressed GST fusion proteins containing various fragments of MAPKK were immobilized and then incubated with the extract of *Xenopus* immature oocytes. After the wash, proteins were eluted by glutathione, and eluted proteins were subjected to SDS–PAGE followed by immunoblotting with anti-*Xenopus* MAPK antibody (αMAPK). A Coomassie blue-stained gel (CBB) shows that comparable amounts of the GST fusion proteins were immobilized on the beads. (**C**) Summary of the activities of various regions of MAPKK which bind to MAPK.

was detected in the precipitate (Figure 3A, lane 3). These data clearly demonstrate the association of MAPK with MAPKK in cells, and extend the previous observations of interaction of these proteins *in vitro* (Zheng and Guan, 1993; Xu *et al.*, 1995).

Activation of the MAPK cascade results in dissociation of the MAPKK–MAPK complex and nuclear translocation of MAPK

If the association of MAPK with MAPKK is a cause of cytoplasmic retention of MAPK in unstimulated cells, nuclear translocation of MAPK should be accompanied by the dissociation of the complex. To test this, we expressed Ras^{val12} in addition to MAPK and HA-MAPKK in cells to activate the MAPK pathway and induce nuclear translocation of MAPK. Then, MAPK became present in the nucleus of the cells (Figure 2C, Ras/WT-KK/MAPK), although HA-MAPKK was present exclusively in the cytoplasm (data not shown). No co-precipitation of MAPK with HA-MAPKK was detected in lysates obtained from the cells expressing MAPK, HA-MAPKK and Ras^{val12} (Figure 3A, lane 8). Similarly, when a constitutively active MAPKK (SDSE-MAPKK) was expressed instead of Ras^{val12}, dissociation of the complex between MAPK and MAPKK was induced (data not shown) and the retention of MAPK in the cytoplasm was released (Figure 2C, SDSE-KK/MAPK). These results, taken together, indicate that nuclear accumulation of MAPK is accompanied by dissociation of a complex between MAPK and MAPKK in the cytoplasm following activation of the MAPK pathway.

Identification of a MAPK-binding region of MAPKK

To define the region of MAPKK responsible for its interaction with MAPK, a series of fusion proteins between portions of MAPKK and GST were bacterially expressed, purified and assayed for the ability to bind to MAPK. The fusion proteins were mixed with the extract prepared from *Xenopus* immature oocytes, and recovered by glutathione–Sepharose. The recovered fractions were examined for the presence of MAPK by immunoblotting with anti-MAPK antibody. The results showed clearly that the N-terminal region of MAPKK (residues 1–32) is the MAPK-binding site (Figure 3B and C). Therefore, the MAPK-binding site (residues 1–32) and the NES sequence (residues 33–44) responsible for the cytoplasmic localization of MAPKK align in tandem in the N-terminal region of MAPKK, outside the kinase catalytic domain.

We have localized the domain of MAPKK that is sufficient for retaining MAPK in the cytoplasm. As already mentioned, injection of an excess amount of the bacterially expressed MAPK protein into the cytoplasm of cells resulted in its nuclear accumulation (Figure 4A). However, when increasing amounts of the fusion protein between GST and residues 1–67 of MAPKK [GST–KK(1–67)] were co-injected, MAPK became excluded from the nucleus and located in the cytoplasm (Figure 4A). Also, co-injection of the fusion protein between GST and 1–60 residues of MAPKK [GST–KK(1–60)] resulted in nuclear exclusion of MAPK (Figure 4B, left panel). Therefore, the peptide comprising the N-terminal sequence of 1–60 residues of MAPKK is sufficient to retain MAPK in the cytoplasm.

NES of MAPKK is necessary for cytoplasmic retention of MAPK

We found that an intact NES sequence of MAPKK is crucial for the ability of MAPKK to retain MAPK in the cytoplasm, because GST–MAPKK (1–32), which lacks

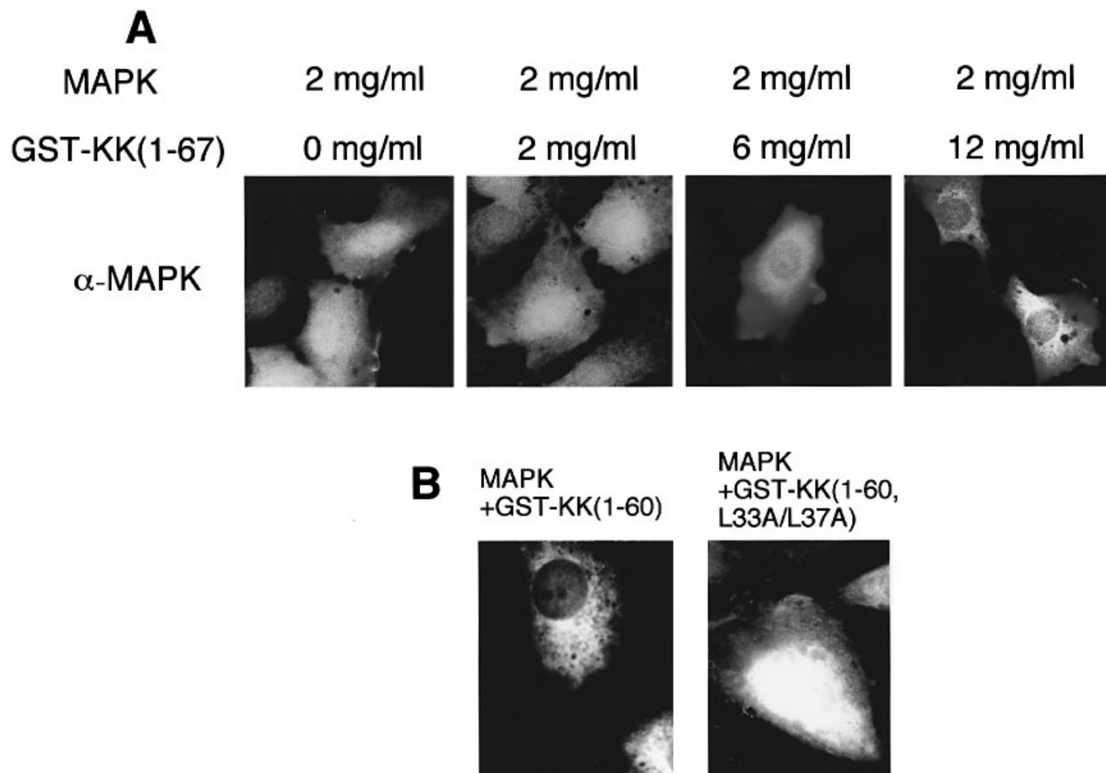

Fig. 4. The N-terminal region of MAPKK encompassing the NES sequence (residues 33–42) and the MAPK-binding site (residues 1–32) is necessary and sufficient to retain MAPK in the cytoplasm. (**A**) Bacterially expressed MAPK protein (2 mg/ml) was injected into the cytoplasm of rat 3Y1 cells together with the fusion protein between GST and the N-terminal portion (residues 1–67) of MAPKK [GST–KK(1–67)] at the indicated concentrations. One hour after injection, cells were fixed and stained with anti-*Xenopus* MAPK antibody. (**B**) The bacterially expressed MAPK with the fusion protein between GST and the N-terminal 1–60 residues of MAPKK [GST–KK(1–60)] (left panel) or with the fusion protein between GST and the same portion of MAPKK with a disrupted NES [GST–KK(1–60, L33A/L37A)] (right panel) was injected into the cytoplasm of cells, and cells were fixed 1 h after injection and stained as above.

the NES sequence (data not shown), and GST–MAPKK (1–60, L33A/L37A), in which the NES activity was destroyed by alanine substitutions of leucines essential for the NES activity, were both unable to retain MAPK to the cytoplasm (Figure 4B, right panel) although both peptides were able to bind to MAPK (Figure 3B and C). Moreover, an NES-disrupted MAPKK mutant, L33A/L37A-MAPKK, was unable to retain MAPK in the cytoplasm (Figure 2D, right panel). These data indicate that the NES is necessary for MAPKK to retain MAPK in the cytoplasm. All the results presented here suggest that MAPK is retained in the cytoplasm through binding to MAPKK whose distribution is restricted to the cytoplasm by its NES.

Specific interaction between MAPK and MAPKK

Finally, we examined the specificity of the interaction between MAPK and MAPKK. To this end, we first examined the effect of expression of other members of the MAPKK superfamily (Davis, 1994; Cano and Mahadevan, 1995), MKK4/SEK1 and MKK3, in addition to MAPKK, on MAPK location. Only MAPKK was able to retain MAPK in the cytoplasm (Figure 5A). Similarly, when we co-expressed with MAPKK other members of the MAPK superfamily (Davis, 1994; Cano and Mahadevan, 1995), p38/HOG1 and SAPK/JNK, in addition to MAPK, only MAPK was retained to the cytoplasm by MAPKK (Figure 5B). Thus, the interaction of classical MAPK with classical MAPKK is specific.

Conclusions

Here we show evidence suggesting that MAPK is localized to the cytoplasm through its specific association with the N-terminal 1–32 residues of MAPKK in unstimulated cells. MAPKK has an NES sequence in residues 33–44 which directs the cytoplasmic location of MAPKK (Fukuda *et al.*, 1996), and the activity of MAPKK in retaining MAPK in the cytoplasm requires the NES of MAPKK. In other words, the NES of MAPKK is required for cytoplasmic retention of MAPK. Thus, MAPKK functions not only as a direct activator of MAPK but also as a cytoplasmic anchoring protein for MAPK. These two functions are fulfilled by two distinct domains of MAPKK (Figure 6). The stimulation of the MAPK pathway may induce dissociation of a complex between MAPK and MAPKK, and then the dissociated, free MAPK may be translocated to the nucleus (Figure 6). Therefore, the dissociation of the complex may be a key step in triggering nuclear translocation of MAPK. The data in Figure 3A show that when about half of MAPK was activated by expression of v-Ras (lane 6), both active and inactive forms of MAPK were dissociated from MAPKK (lane 8), and thus suggest that after activation of the MAPK pathway dissociation of the complex between MAPK and MAPKK occurs irrespective of the phosphorylation state of MAPK. This is consistent with the findings that a kinase-deficient mutant (Gonzalez *et al.*, 1993) or non-phosphorylated mutants (Lenormand *et al.*, 1993) of MAPK can be translocated to the nucleus by serum

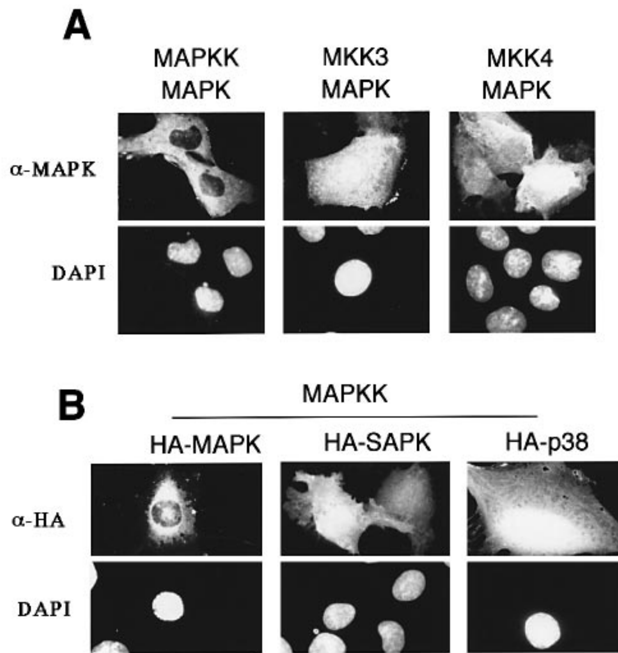

Fig. 5. Specific interaction between MAPK and MAPKK. (**A**) A mixture of a *Xenopus* MAPK plasmid and either a MAPKK plasmid, a MKK3 plasmid (Moriguchi *et al.*, 1996) or a MKK4 plasmid was injected into the nuclei of 3Y1 cells. Cells were fixed and stained with anti-MAPK antibody. (**B**) A mixture of *Xenopus* MAPKK plasmid and either an HA-MAPK plasmid, an HA-SAPK plasmid or an HA-p38 plasmid (Moriguchi *et al.*, 1996) was injected into the nuclei of 3Y1 cells. Cells were fixed and stained with anti-HA monoclonal antibody.

stimulation or by expressing constitutively active MAPKK (our unpublished data). Thus, the dissociation of the complex between MAPKK and MAPK may not be achieved simply through phosphorylation of MAPK. Since it has been reported previously that MAPK can phosphorylate MAPKK on threonine residues (Matsuda *et al.*, 1993; Brunet *et al.*, 1994; Gotoh *et al.*, 1994; Saito *et al.*, 1994), there is a possibility that the feedback phosphorylation of MAPKK by MAPK may be involved in the control of complex formation. The molecular mechanisms inducing the dissociation of the complex remain to be clarified.

Materials and methods

Cell culture and microinjection

Rat 3Y1 cells were plated onto CELLocate coverslips (Eppendorf) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 0.2 mg/ml kanamycin) for 2 days, and then placed in starvation medium (DMEM without FCS) for 24–48 h before microinjection. Microinjection was performed using an IM-188 microinjection apparatus (Narishige). Cells cultured on marked areas of CELLocate coverslips were microinjected with the samples.

Cell staining

The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min, and then permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. The coverslips were incubated with primary antibodies at room temperature for 1 h, and then with the appropriate secondary antibodies. The primary antibodies and dilutions used here were a rabbit antiserum to *Xenopus* MAPK (1:100 dilution), a rabbit antibody to *Xenopus* MAPKK (1:100 dilution) and a monoclonal antibody to HA (12CA5; 20 μg/ml). Injected areas of cells were finally mounted in Mowiol and examined using a Zeiss Axiophot.

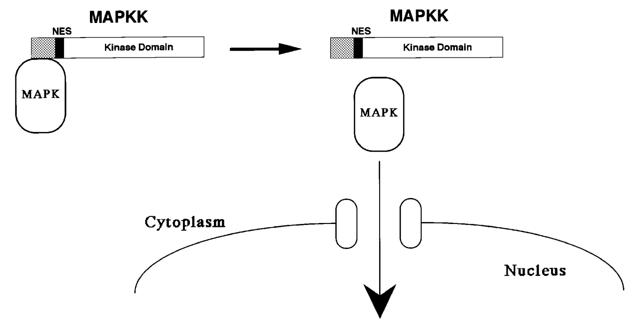

Fig. 6. A proposed model explaining the mechanism by which the subcellular distribution of MAPK is determined.

Transfection and immunoprecipitation

COS-7 cells (1×10^5 cells per well) were seeded in 6-well plates in DMEM supplemented with 10% FCS for 16 h before transfection. The total amount of DNA in the transfection was 3 μg per well. The plasmids were mixed with lipofectamine (GIBCO) in serum-free Opti-MEM (GIBCO) and cells were transfected. Five hours after transfection, the cells were incubated in medium containing 10% FCS for an additional 36 h, and further incubated in serum-free DMEM for 6 h before extraction. Cells were washed with ice-cold PBS, and scraped into an ice-cold extraction buffer (100 μl per well) (E-buffer; 20 mM HEPES–NaOH, pH 7.4, 2 mM $MgCl_2$, 2 mM EGTA), and the cell lysates were centrifuged at 15 000 *g* for 30 min. The supernatant was subjected to immunoprecipitation with antibody to HA. Briefly, a portion (150 μl) of the supernatant was mixed with 7.5 μl of the antibody (α-HA; 5.0 mg/ml) and 50 μl of protein A–Sepharose beads (Pharmacia) at 4°C for 1 h, and the mixture was poured into the column (Muromac). The columns were washed with 200 μl of the E-buffer three times, and then eluted with an elution buffer (100 mM Gly-HCl, pH 2.2). The eluted fraction was subjected to immunoblotting with anti-*Xenopus* MAPKK antibody or anti-*Xenopus* MAPK antibody.

Antibodies

A rabbit anti-*Xenopus* MAPKK antibody was raised against the N-terminal peptide (residues 1–16) of *Xenopus* MAPKK (Kosako *et al.*, 1992). An anti-*Xenopus* MAPK antibody was raised in rabbit against bacterially expressed recombinant His-tagged MAPK. This antibody recognized *Xenopus* MAPK, but not mammalian MAPKs (data not shown).

DNA construction

The bacterial expression plasmids expressing fusion proteins between GST and different subfragments (residues 1–134, 1–67, 1–32, 1–23 and 23–134) of *Xenopus* MAPKK were constructed previously (Kosako *et al.*, 1996). The N-terminal region (1–60) of *Xenopus* MAPKK was amplified by PCR with a 5′ primer, 5′-CCGGGGATCCATGCCTA-AAAAGAAGCCTAC-3′ and a 3′ primer, 5′-GGCCGAATTCAACTTT-CTGCTTCTGGGTGA-3′. A *Bam*HI–*Eco*RI fragment was cloned into pGEX-2T. For construction of GST–KK(1–60, L33A/L37A), an amplification of the N-terminal region of MAPKK was carried out by PCR with the template L33A/L37A-MAPKK-pcDL-SRα457 (Fukuda *et al.*, 1996) instead of wild-type MAPKK cDNA. An HA tag was introduced into the *Bgl*II–*Eco*RI sites of an expression vector pcDL-SRα457 by ligating with the oligonucleotides 5′-GATCGCCGCCACCATGTACC-CATACGACGTCCCAGATTACGCTCAGATCTG-3′ and 5′-AATTC-AGATCTGAGCGTAATCTGGGACGTCGTATGGGTACATGGTGGC-GGC-3′ yielding pSRα-HA2. To obtain HA-tagged *Xenopus* MAPK, the open reading frame (ORF) of *Xenopus* MAPK was cloned into pSRα-HA2. The mutagenesis of Ser218 to Asp and Ser222 to Glu in *Xenopus* MAPKK was performed using a mutagenic primer 5′-GCGGGCAACTCATAGACGACATGGCAAATGAGTTTGTTGGGA-CAAGATC-3′ to yield SDSE-MAPKK. The ORF of SDSE-MAPKK was amplified by PCR with a 5′ primer 5′-CACTAGATCTCAACA-TGCCTAAAAAGAA-3′ and a 3′ primer 5′-GCCAAGATCTCTCACA-CTCCGGCGGCAT-3′, which produce *Bgl*II sites at both ends of the mutant of MAPKK. Each *Bgl*II fragment was cloned into pcDL-SRα457. Rat SAPKα and mouse MKK4 were subcloned into a pSRα-HA vector to generate pSRα-HA-SAPK and pSRα-HA-MKK4, respectively.

Preparation of recombinant proteins

Histidine (His)-tagged *Xenopus* MAPK was expressed and purified by Ni^{2+}-chelate chromatography (Fukuda *et al.*, 1995). v-Ha-Ras was expressed in and isolated from *Escherichia coli* as previously described (Hattori *et al.*, 1992). An N-terminal truncated STE11 protein (residues 370–717; ΔN-STE11) was expressed in bacteria as a GST fusion protein and purified. The fusion proteins between GST and various portions of MAPKK were bacterially expressed and purified on glutathione–Sepharose (Pharmacia) as previously described (Kosako *et al.*, 1996). They were concentrated using Centricon-30 (Amicon). The protein concentration was determined by the Bradford assay (Bio-Rad). Since the concentrated proteins were not so viscous, we were able to inject them into cells without clogging the needles.

In vitro binding assay

Xenopus immature oocytes were extracted in the E-buffer, and centrifuged at 100 000 *g* for 1 h. The resultant supernatant was used as the source of MAPK. This supernatant was mixed with the GST protein or the GST–MAPKK derivatives (final concentrations, 300 μg/ml each) at 4°C for 1 h, and poured into the column (Muromac). The columns were washed with 200 μl of the E-buffer three times, and then eluted with a buffer (10 mM glutathione, 20 mM Tris–HCl, pH 8.0). The eluted fraction was subjected to SDS–PAGE and then to immunoblotting with anti-*Xenopus* MAPK antibody.

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