Nuclear Export of MAP Kinase (ERK) Involves a MAP Kinase Kinase (MEK)-dependent Active Transport Mechanism

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Abstract. In response to extracellular stimuli, mitogen-activated protein kinase (MAPK, also known as ERK), which localizes to the cytoplasm in quiescent cells, translocates to the nucleus and then relocalizes to the cytoplasm again. The relocalization of nuclear MAPK to the cytoplasm was not inhibited by cycloheximide, confirming that the relocalization is achieved by nuclear export, but not synthesis, of MAPK. The nuclear export of MAPK was inhibited by leptomycin B (LMB), a specific inhibitor of the nuclear export signal (NES)-dependent transport. We have then shown that MAP kinase kinase (MAPKK, also known as MEK), which mostly localizes to the cytoplasm because of its having NES, is able to shuttle between the cytoplasm and the nucleus constantly. MAPK, when injected into the nucleus, was rapidly exported from the nucleus by coinjected wild-type MAPKK, but not by the NES-disrupted MAPKK. In addition, injection of the fragment corresponding to the MAPK-binding site of MAPKK into the nucleus, which would disrupt the binding of MAPK to MAPKK in the nucleus, significantly inhibited the nuclear export of endogenous MAPK. Taken together, these results suggest that the relocalization of nuclear MAPK to the cytoplasm involves a MAPKK-dependent, active transport mechanism.

Key words: leptomycin B • MAP kinase • nuclear export • phosphorylation • signal transduction

Introduction

The mitogen-activated protein kinase (MAPK, also known as ERK)[1] cascade is a central signal transduction pathway that is activated by growth factors, and is known to be involved in diverse cellular functions (Sturgill and Wu, 1991; Thomas, 1992; Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Marshall, 1994; Lewis et al., 1998; Robinson and Cobb, 1997). In response to a wide variety of extracellular stimuli, three classes of protein kinases, MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK, also known as MEK) and MAPK, are sequentially activated. Activation of MAPK leads to its translocation from the cytoplasm to the nucleus, where MAPK phosphorylates and activates several nuclear targets such as transcription factors (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993). Nuclear localization of MAPK is transient, however, and MAPK relocalizes to the cytoplasm to prepare for the next activation.

To date, a number of reports have addressed regulatory mechanisms of the nuclear translocation of MAPK. We have previously demonstrated that MAPKK (MEK1 or MEK2), a direct activator of MAPK (ERK1 or ERK2), acts as a cytoplasmic anchor of MAPK (Fukuda et al., 1997a). MAPKK apparently localizes to the cytoplasm irrespective of its activation state (Lenormand et al., 1993; Zheng and Guan, 1994; Moriguchi et al., 1995), and this is due to its nuclear export signal (NES) at its NH_2 terminus (Fukuda et al., 1996). Upon stimulation, MAPK dissociates from MAPKK (Fukuda et al., 1997a). The dissociation requires tyrosine phosphorylation of MAPK by MAPKK (Adachi et al., 1999). Khokhlatchev et al. (1998) have demonstrated that homodimerization of MAPK, which is induced by phosphorylation of MAPK, is an important mechanism for nuclear translocation of MAPK.

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[1]*Abbreviations used in this paper:* CHX, cycloheximide; DAPI, 4′,6-diamidino-2-phenylindole; LMB, leptomycin B; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; NES, nuclear export signal; TPA, 12-*O*-tetra decanoyl phorbol myristate acetate.

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We have then shown that dimeric MAPK enters the nucleus by using an active transport mechanism, whereas MAPK can also enter the nucleus by passive diffusion as a monomer (Adachi et al., 1999). Moreover, Lenormand et al. (1998) proposed that activation of the MAPK cascade leads to neosynthesis of nuclear anchoring protein for MAPK, which seems to be required for sustained nuclear retention of MAPK. MAPK then must relocalize to the cytoplasm again to prepare for the next stimulation. However, so far, there have been no reports directly examining how MAPK relocalizes to the cytoplasm after its nuclear entry.

Here we first show evidence that the relocalization of MAPK to the cytoplasm is achieved by nuclear export of MAPK. Then, we have demonstrated that the nuclear export of MAPK is inhibited by LMB, a specific inhibitor of the NES-dependent transport. MAPKK, which mostly localizes to the cytoplasm because of its NES, is shown to be able to shuttle between the cytoplasm and the nucleus constantly under normal conditions. While MAPKK is exported from the nucleus by the NES-dependent active transport, its nuclear entry seems to be achieved by passive diffusion, since β-gal-MAPKK, in which β-galactosidase was fused to the NH_2 terminus of MAPKK to make MAPKK too large to pass through a nuclear pore by diffusion, could not enter the nucleus even in the presence of LMB. Moreover, our result demonstrates that the export of nuclear injected MAPK from the nucleus was rapidly induced by nuclear coinjection of wild-type MAPKK, but not by that of the NES-disrupted MAPKK. In addition, nuclear injection of the NH_2-terminal portion of MAPKK, which contains the MAPK-binding site, inhibited the nuclear export of MAPK. Therefore, it is likely that MAPK is exported from the nucleus to the cytoplasm by MAPKK. Taken together, our findings suggest that MAPKK not only acts as a cytoplasmic anchor of MAPK but also mediates nuclear export of MAPK and, therefore, may be a central regulator of subcellular localization of MAPK.

Materials and Methods

Cell Culture and Microinjection

Xenopus A6 cells and rat 3Y1 cells plated onto coverslips were cultured in Leibovitz's L-15 medium or DME supplemented with 10% FCS and antibiotics (100 U/ml penicillin and 0.2 mg/ml kanamycin), respectively. ΔB-Raf:ER cells (Pritchard et al., 1995) were cultured in DME without phenol red supplemented with FCS, antibiotics, and 25 mM Hepes (pH. 7.4). Microinjection was performed using an IM-188 microinjection apparatus (Narishige). Samples were dissolved in injection buffer (20 mM Hepes-KOH, pH 7.4, 120 mM KCl).

Cell Staining

Cells were fixed by the direct addition of formaldehyde (final concentration 3.7%) to the cell culture medium and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. The coverslips were incubated with primary antibodies at 4°C overnight, and then with the appropriate secondary antibodies at room temperature for 1 h. The primary antibodies and dilutions used here were a rabbit antiserum to *Xenopus* MAPK (Fukuda et al., 1997a; 1:200 dilution), a rabbit anti-ERK1 antibody (K-23; Santa Cruz; 1:100) and a mouse monoclonal anti-HA antibody (12CA5, 1:200). Cells were finally mounted in Mowiol and examined using a Zeiss Axiophoto.

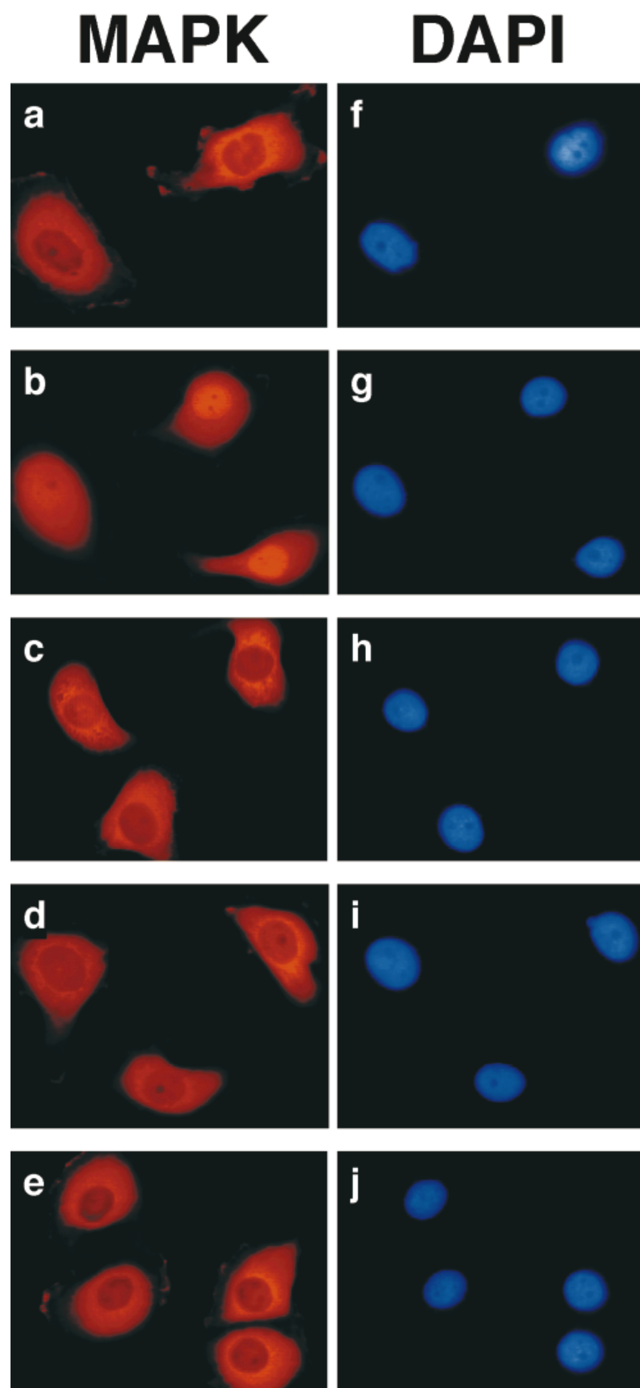
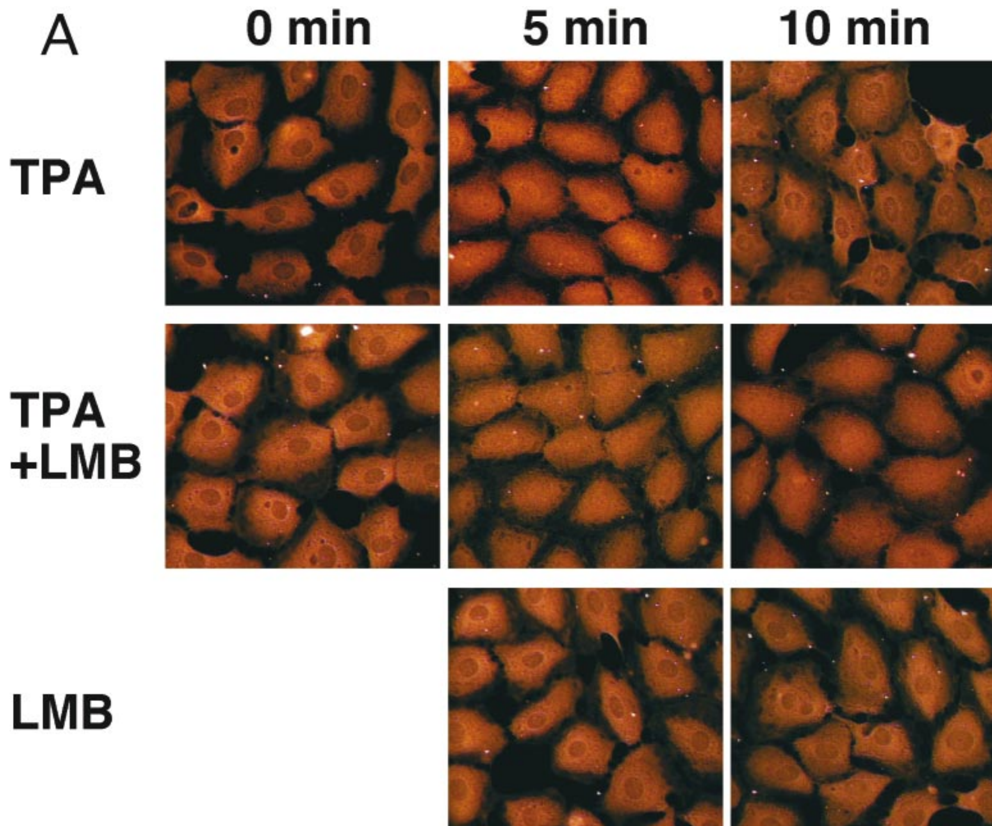


Figure 1. Relocalization of nuclear MAPK to the cytoplasm is mediated by nuclear export. Staining images of cells with anti-*Xenopus* MAPK antibody (a–e) or DAPI (f–j) are shown. A6 cells were serum starved for 36 h (a and f), and then stimulated with 10% FCS for 4 h (b and g). Then cells were treated with CHX (50 μg/ml) for 10 min, followed by deprivation of serum for 10 min in the presence of CHX (d and i), or cells were treated with control buffer for 10 min and then deprived of serum for 10 min in the presence of the buffer (c and h). Incubation of cells with CHX or buffer for 10 min alone did not change subcellular distribution of MAPK (data not shown). In agreement with the result of Lenormand et al. (1998), stimulation of cells with 10% FCS for 4 h in the presence of CHX (50 μg/ml) did not induce nuclear accumulation of MAPK (e and j), indicating that CHX at this concentration is effective. Experiments were performed twice with similar results.



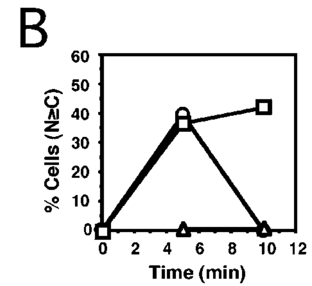


Figure 2. LMB inhibits the nuclear export of MAPK. 3Y1 cells were serum starved for 36 h. Then, the cells were stimulated with TPA alone (500 ng/ml, upper panels in A, circles in B). LMB (0.4 ng/ml) was added 5 min after the addition of TPA (middle panels in A, squares in B), or only LMB without TPA treatment was added (lower panels in A, triangles in B). Cells were fixed, and then stained with anti-ERK1 antibody. The representative images are shown in A. (B) Quantification of the data from a representative experiment. The percentages of cells in which nuclear MAPK staining was stronger than, or equal to, cytoplasmic MAPK staining are shown in each condition. 86–116 cells were examined in each condition. Experiments were performed four times with similar results.

DNA Construction and Transfection

A BamHI-BglII fragment of *Escherichia coli* β-galactosidase gene (Adachi et al., 1999) was subcloned into the BglII site of pSRαHA1 to yield pSRαHA-β-gal. A BglII fragment of *Xenopus* MAPKK (Adachi et al., 1999) is cloned into the BglII site of pSRαHA-β-gal, yielding pSRαHA-β-gal-MAPKK. A BglII fragment of LA MAPKK obtained from pSRαHA-LA MAPKK (Fukuda et al., 1996) was subcloned into pET-28a (Novagen) to obtain pET-28a LA MAPKK.

Preparation of Recombinant Proteins

The fusion protein between GST and the NH_2-terminal portion of MAPKK (residues 1–60) with a disrupted NES (GST KK1-60 LA) was prepared as described (Fukuda et al., 1997a). His-tagged MAPK and His-tagged wild-type and NES-disrupted (LA) MAPKK were prepared as described (Gotoh et al., 1994).

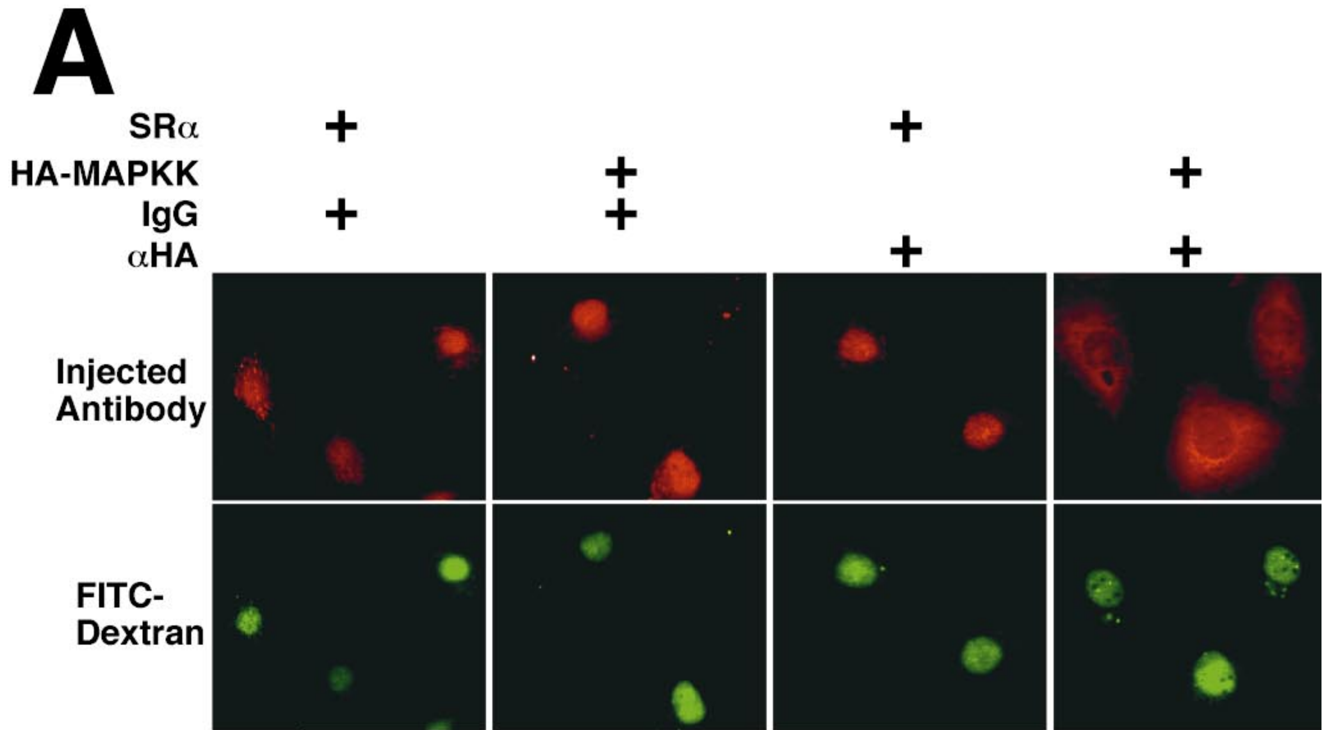
Results

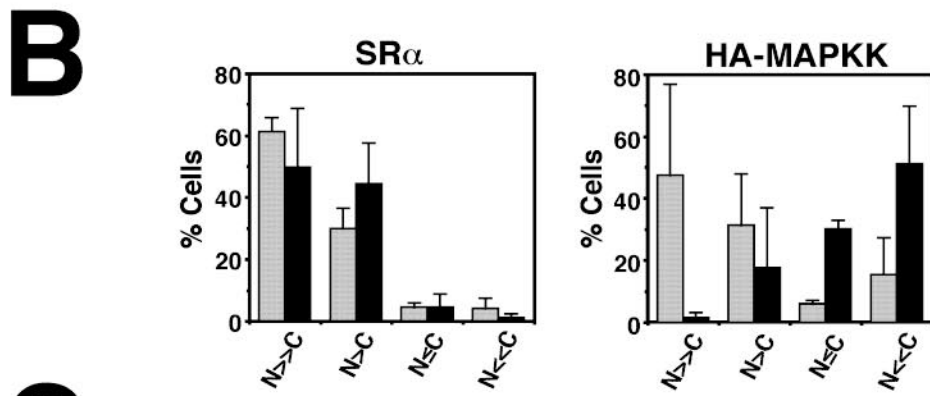
Relocalization of Nuclear MAPK to the Cytoplasm Is Mediated by a Nuclear Export Mechanism

Nuclear translocation of MAPK is transient, although the duration time in the nucleus varies depending on the cell types and the stimuli used. Thus, nuclear MAPK should relocalize to the cytoplasm. However, it is also possible that nuclear MAPK is degraded and newly synthesized MAPK appears in the cytoplasm. To test this possibility, we examined the effect of a protein synthesis inhibitor cycloheximide (CHX) on the relocalization of MAPK to the cytoplasm. In A6 cells, nuclear accumulation of MAPK (*Xenopus* MPK1 = ERK2) is most strongly induced after 4 h of stimulation with 10% FCS. Subsequent serum removal triggers appearance of MAPK in the cytoplasm and disappearance of nuclear MAPK. CHX was added to the cultures 4 h after stimulation with serum. After a 10-min incubation, cells were deprived of serum in the presence of CHX, and then the cells were fixed 10 min later. The indirect immunostaining of endogenous MAPK demonstrated that MAPK relocalized to the cytoplasm even in the presence of CHX (Fig. 1). Therefore, relocalization of nuclear MAPK to the cytoplasm is mediated by a nuclear export mechanism, not by synthesis of MAPK in the cytoplasm.

LMB Inhibits Nuclear Export of MAPK

We then tested the effect of LMB on the nuclear export of MAPK. In 3Y1 cells, MAPK translocated to the nucleus within 5 min after stimulation with TPA, and after 10 min MAPK was exported from the nucleus (Fig. 2, TPA). When LMB was added 5 min after TPA stimulation (i.e., when nuclear translocation of MAPK was achieved), subsequent nuclear export of MAPK was strongly inhibited (Fig. 2, TPA+LMB). Similar results were obtained in A6 cells (data not shown). As LMB is a specific inhibitor of NES receptor (Fornerod et al., 1997; Fukuda et al., 1997b;





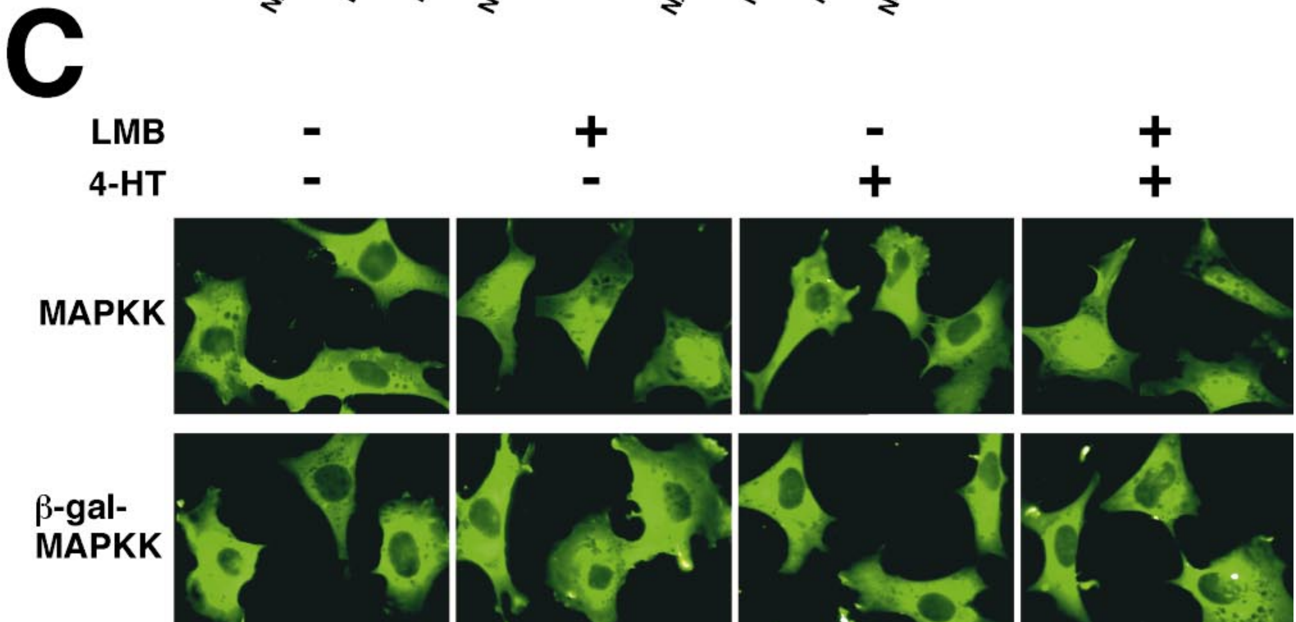


Figure 3.

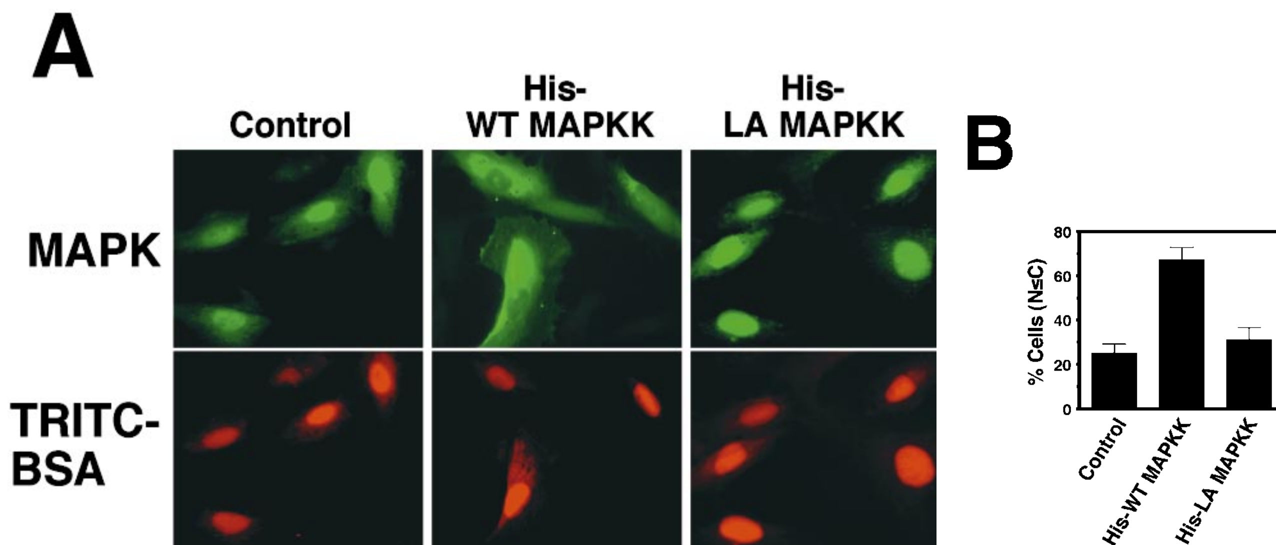


Figure 4. Nuclear export of MAPK protein is induced by nuclear coinjection of wild-type MAPKK, but not by that of NES-disrupted MAPKK. The nuclei of 3Y1 cells were injected with TRITC-BSA and His-tagged MAPK protein (5.0 mg/ml) without (Control) or with His-tagged wild-type MAPKK protein (5.0 mg/ml; His-WT MAPKK) or His-tagged, NES-disrupted MAPKK protein (5.0 mg/ml; His-LA MAPKK). 5 min after injection, cells were fixed and stained with anti-MAPK antibody. The representative images are shown in A. (B) Quantification of the data from three independent experiments. The percentages of cells which showed stronger MAPK fluorescence intensity in the cytoplasm than in the nucleus are shown in each condition. 76–272 cells were examined in each condition in one experiment.

Ossareh-Nazari et al., 1997; Wolff et al., 1997), it is suggested that the nuclear export of MAPK is mediated by the NES-dependent active transport mechanism.

MAPKK Shuttles between the Cytoplasm and the Nucleus

Because MAPK does not seem to have an NES-like sequence, we thought that some other NES-possessing molecules may convey MAPK from the nucleus to the cytoplasm. As MAPKK has an NES at its NH_2 terminus (Fukuda et al., 1996) and specifically binds to MAPK (Fukuda et al., 1997a), we hypothesized that MAPKK is a possible carrier of MAPK. To perform this function, however, MAPKK must enter the nucleus at least transiently, although MAPKK mostly localizes to the cytoplasm (Lenormand et al., 1993; Zheng and Guan, 1994; Moriguchi et al., 1995). To examine whether MAPKK is able to shuttle between the cytoplasm and the nucleus, we carried out the following experiment. In quiescent 3Y1 cells, HA-tagged MAPKK was expressed by the plasmid injection, and anti-HA antibody was injected into the nucleus. Then, location of the injected anti-HA antibody was examined. In the absence of HA-MAPKK, the anti-HA antibody remained in the nucleus, while in the presence of the expressed HA-MAPKK, most of anti-HA antibody became detected in the cytoplasm (Fig. 3, A and B). These results are interpreted as indicating that HA-MAPKK expressed in the cytoplasm enters the nucleus where HA-MAPKK binds to anti-HA antibody, and then the HA-MAPKK/anti-HA antibody complex is exported from the nucleus. Therefore, MAPKK has now been shown to shuttle be-

Figure 3. MAPKK shuttles between the cytoplasm and the nucleus. (A and B) 3Y1 cells were serum starved for 48 h. Then, the nuclei were injected with anti-HA antibody (αHA, 20.0 mg/ml) or control IgG (IgG, 20.4 mg/ml) together with the plasmid harboring HA-tagged MAPKK (HA-MAPKK, 150 μg/ml) or the corresponding amount of an empty vector (SRα), all together with FITC-labeled dextran (FITC-Dextran, 800 μg/ml). 5 or 16 h after injection, cells were fixed and stained with Cy3-labeled anti–mouse IgG. The typical images at 16 h after injection are shown in A. (B) Quantification of the data from two independent experiments for nuclear injection of anti-HA antibody (αHA) with SRα or the HA-MAPKK plasmid. The cells were classified into four categories in terms of location of the injected αHA antibody (= Cy3 staining intensity): $N >> C$, staining intensity in the nucleus (N) is much stronger than that in the cytoplasm (C); $N > C$, N is stronger than C; $N \leq C$, C is equal to or stronger than N; $N << C$, C is much stronger than N (the αHA is almost completely exported from the nucleus). 56–295 cells were examined in each condition in one experiment. Grey bars, 5 h after injection; black bars, 16 h after injection. Under the conditions used, expression of HA-MAPKK, which was revealed by anti-HA staining in another series of experiments, occurred in almost all the plasmid-injected cells within 4 h. The export of the nuclear injected αHA from the nucleus was clearly detected even 5 h after the nuclear injection of the HA-MAPKK plasmid (see this figure), so the shuttling of HA-MAPKK may occur frequently. (C) ΔB-Raf:ER cells (Pritchard et al., 1995) were transfected with either SRαHA-MAPKK or SRαHA-β-gal-MAPKK together with pCDNA3 MAPK. 16 h later cells were treated with LMB (LMB, 20 ng/ml) and/or 4-hydroxytamoxifen (4-HT, 1 μM), which activates ΔB-Raf, for 5 h. Cells were then fixed and stained with anti-HA antibody. Experiments were performed twice with similar results.

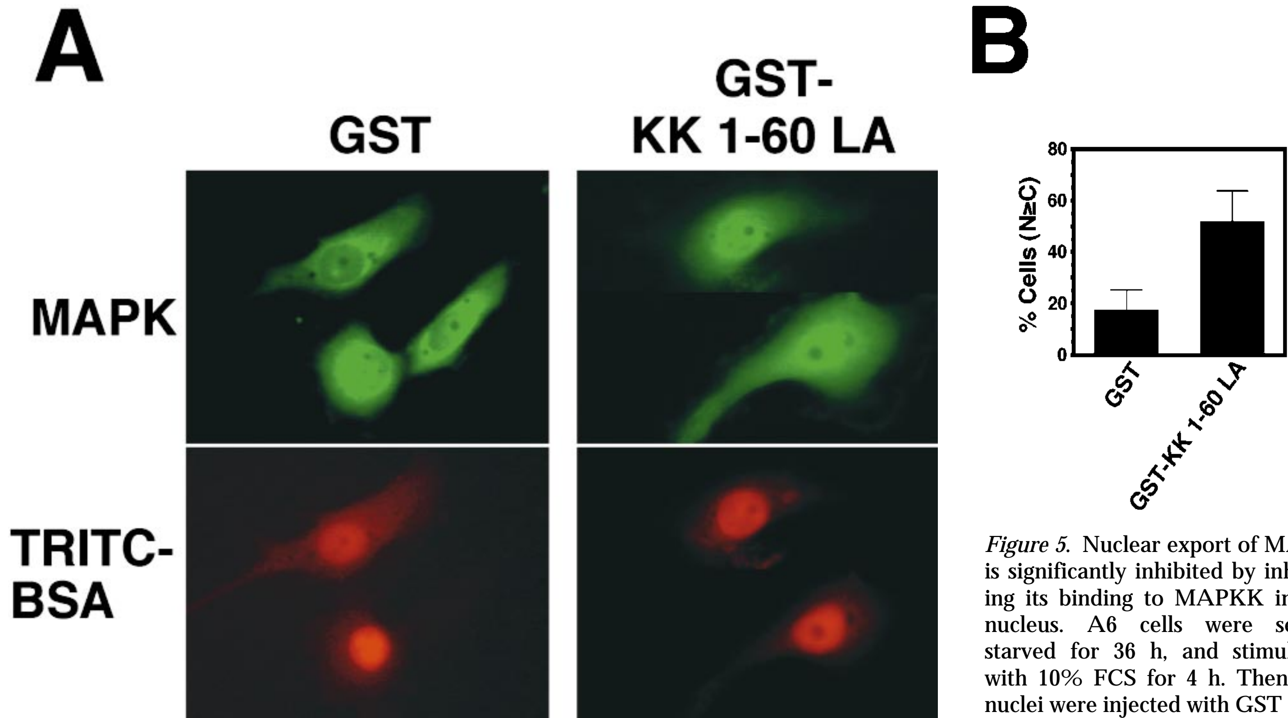


Figure 5. Nuclear export of MAPK is significantly inhibited by inhibiting its binding to MAPKK in the nucleus. A6 cells were serum starved for 36 h, and stimulated with 10% FCS for 4 h. Then, the nuclei were injected with GST (15.0 mg/ml) or the GST fusion protein of the NH_2-terminal 1–60 residues of MAPKK with a disrupted NES (GST-KK 1-60 LA, 16.0 mg/ml) together with TRITC-BSA. Then, the culture medium was replaced by the serum-free medium. 10 min after the serum removal, cells were fixed and stained with anti-MAPK antibody. The typical images are shown in A. (B) Quantification of the data from three independent experiments. The percentages of cells which showed stronger MAPK staining in the nucleus than in the cytoplasm are shown in each condition. 133–262 cells were examined in each condition in one experiment.

tween the cytoplasm and the nucleus constantly. Moreover, our finding with the indirect immunofluorescent cell staining method that nuclear entry of MAPKK can be clearly detected as early as 10 min after LMB treatment of the cells (Fukuda et al., 1997b) is consistent with the idea that MAPKK is shuttling between the cytoplasm and the nucleus constantly and suggests that the shuttling is occurring rapidly.

Previous studies have suggested that nuclear entry of MAPKK is enhanced in a stimulus-dependent manner, although in normal conditions it is not apparent since MAPKK is immediately exported to the cytoplasm by means of its NES (Jaaro et al., 1997; Tolwinski et al., 1999). This possible mechanism is consistent with our idea of MAPKK shuttling. To examine the possible involvement of active nuclear import mechanisms in the regulated nuclear entry of MAPKK, we used β-gal-MAPKK in which β-galactosidase is fused to the NH_2 terminus of MAPKK to make MAPKK too large to pass through a nuclear pore by diffusion. As shown in Fig. 3 C, β-gal-MAPKK did not translocate to the nucleus even in the presence of LMB and the stimulus that activates the MAPK cascade. Therefore, the regulated nuclear entry of MAPKK, if present, may not be achieved through active nuclear import mechanisms. In fact, cooling of cells or deprivation of ATP induced nuclear entry of MAPKK (Adachi et al., 1999; data not shown). This also supports our idea that MAPKK enters the nucleus constantly by passive diffusion and is always exported actively by the NES-dependent, active mechanism.

Nuclear Injection of MAPKK with MAPK Induces Nuclear Export of MAPK

If MAPKK indeed takes MAPK out of the nucleus, then nuclear export of MAPK should be accelerated by the presence of excess MAPKK in the nucleus. To test this idea, we injected recombinant MAPK protein into the nuclei of 3Y1 cells together with or without recombinant MAPKK protein and then examined the subcellular distribution of injected MAPK protein 5 min after injection. When injected alone, MAPK stayed in the nucleus (Fig. 4, Control). In contrast, coinjection of wild-type MAPKK significantly induced the nuclear export of MAPK (Fig. 4, His-WT MAPKK). Importantly, NES-disrupted MAPKK did not induce the nuclear export of MAPK at all (Fig. 4, His-LA MAPKK). These results clearly demonstrate that MAPKK is able to carry MAPK out of the nucleus by using the NES of MAPKK.

Nuclear Export of MAPK Is Blocked by Inhibition of the Binding between MAPKK and MAPK in the Nucleus

If MAPKK is necessary for nuclear export of MAPK, then the inhibition of the binding between MAPK and MAPKK in the nucleus would result in the blockade of the nuclear export of MAPK. To inhibit the binding, we injected a peptide fragment (GST-KK 1-60 LA) that corresponds to the MAPK-binding site of MAPKK (the fusion

protein between GST and the NH_2-terminal region of MAPKK, residues 1–60, in which the NES is disrupted) into the nuclei of A6 cells. It has been shown that MAPK binds to the residues 1–32 of MAPKK (Bardwell et al., 1996; Fukuda et al., 1997a). Thus, microinjection of the fragment into the nuclei was performed 4 h after stimulation with serum. And then, 10 min after serum removal, the cells were fixed. The indirect immunostaining demonstrated that injection of GST-KK 1-60 LA, but not that of GST, inhibited the nuclear export of endogenous MAPK (Fig. 5, A and B). Although we cannot exclude the possibility that some other molecule which can bind to the MAPKK binding region of MAPK might be a carrier of MAPK, the above result strongly suggests that the nuclear export of MAPK is mediated by MAPKK.

Discussion

This study is the first to deal with the mechanism of the nuclear export process of MAPK, and suggests that nuclear export of MAPK is mediated by MAPKK. Our results show that (a) relocalization of nuclear MAPK to the cytoplasm is achieved by a nuclear export mechanism; (b) LMB, a specific inhibitor of NES-dependent active transport, inhibits nuclear export of MAPK; (c) MAPKK, which possesses an NES, shuttles between the cytoplasm and the nucleus; (d) coinjection of MAPKK with MAPK into the nucleus rapidly induces the nuclear export of MAPK; and (e) inhibition of the binding between MAPKK and MAPK in the nucleus prevents the nuclear export of MAPK. From these results, we suggest that MAPKK has a role in conveying MAPK from the nucleus to the cytoplasm, although we cannot rule out completely the possibility that some other molecule with an NES is a carrier of MAPK.

Importantly, if MAPKK is a carrier of MAPK for its export, dephosphorylated MAPK is selectively exported to the cytoplasm, because only dephosphorylated MAPK binds to MAPKK (Adachi et al., 1999). Consistent with this, our immunostaining experiment with anti-active MAPK antibody and anti-MAPK antibody showed that active MAPK did not relocalize to the cytoplasm after the nuclear entry in response to TPA stimulation in 3Y1 cells and became deactivated in the nucleus, while the bulk of MAPK relocalized to the cytoplasm (data not shown). Moreover, the report of Khokhlatchev et al. (1998) demonstrated that thiophosphorylated MAPK, which was injected into the nucleus remained in the nucleus for a long time, whereas unphosphorylated MAPK relocalized to the cytoplasm immediately. This is also in agreement with our model. This model also suggests that nuclear export of MAPK does not have a role in shutting off the MAPK pathway, because subcellular distribution of only inactive MAPK can be controlled by the export mechanism. So nuclear export of MAPK may function to restore cytoplasmic pool of activatable MAPK for the next stimulation and, therefore, to ensure sustained and continuous activation of the MAPK cascade.

In summary, we have demonstrated that nuclear export of MAPK involves a MAPKK-dependent, active transport mechanism. Because inactivated MAPK preferentially binds to MAPKK, MAPK, once deactivated in the nucleus, would be rapidly excluded from the nucleus. Therefore, this mechanism may work to localize inactive MAPK to the cytoplasm selectively, thus making the cells respond to extracellular stimuli rapidly and continuously.

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