Two co-existing mechanisms for nuclear import of MAP kinase: passive diffusion of a monomer and active transport of a dimer

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In response to extracellular stimuli, mitogen-activated protein kinase (MAPK, also known as ERK) translocates from the cytoplasm to the nucleus. MAP kinase kinase (MAPKK, also known as MEK), which possesses a nuclear export signal (NES), acts as a cytoplasmic anchor of MAPK. Here we show evidence that tyrosine (Tyr190 in *Xenopus* MPK1/ERK2) phosphorylation of MAPK by MAPKK is necessary and sufficient for the dissociation of the MAPKK–MAPK complex, and that the dissociation of the complex is required for the nuclear translocation of MAPK. We then show that nuclear entry of MAPK through a nuclear pore occurs via two distinct mechanisms. Nuclear import of wild-type MAPK (mol. wt 42 kDa) was induced by activation of the MAPK pathway even in the presence of wheat germ agglutinin or dominant-negative Ran, whereas nuclear import of β -galactosidase (β -gal)-fused MAPK (mol. wt 160 kDa), which occurred in response to stimuli, was completely blocked by these inhibitors. Moreover, while a dimerization-deficient mutant of MAPK was able to translocate to the nucleus upon stimulation, this mutant MAPK, when fused to β -gal, became unable to enter the nucleus. These results suggest that monomeric and dimeric forms of MAPK enter the nucleus by passive diffusion and active transport mechanisms, respectively.

Keywords: nuclear translocation/signal transduction/tyrosine phosphorylation

Introduction

A variety of signals accepted at the cell-surface receptors must be properly integrated or transmitted to the nucleus to induce the appropriate gene expression. The mitogen-activated protein kinase (MAPK) cascade is among many pathways involved in these signal transductions, consisting of the array of sequential activation of MAP kinase kinase (MAPKKK, also known as MEKK), MAP kinase kinase (MAPKK, also known as MEK) and MAPK (also known as ERK), and plays important roles in several cellular functions (Sturgill and Wu, 1991; Thomas, 1992; Blenis, 1993; Davis, 1993; Nishida and Gotoh, 1993; Marshall, 1994; Robinson and Cobb, 1997; Lewis *et al.*, 1998). It has been reported that upon activation of the MAPK cascade, classical MAPKs, ERK1 and ERK2, are

phosphorylated by MEKs (MEK1 or 2) on threonine and tyrosine residues (Thr188 and Tyr190 in *Xenopus* MAPK=MPK1/ERK2; Payne *et al.*, 1991), and translocate from the cytoplasm to the nucleus (Chen *et al.*, 1992; Gonzalez *et al.*, 1993; Lenormand *et al.*, 1993), where MAPKs phosphorylate several nuclear targets such as transcription factors. Not only activation, but also the nuclear translocation of MAPKs, appears to be a prerequisite for proper cellular responses (Brunet *et al.*, 1999).

In contrast to MAPKs, classical MAPKKs, MEK1 (MKK1) and MEK2 (MKK2) always localize to the cytoplasm (Lenormand *et al.*, 1993; Zheng and Guan, 1994; Moriguchi *et al.*, 1995). We have previously shown that the cytoplasmic localization of MAPKK is achieved by its nuclear export signal (NES) at the N-terminus (Fukuda *et al.*, 1996). Moreover, the cytoplasmic localization of MAPK in quiescent cells is mediated by its specific binding to MAPKK, and nuclear translocation of MAPK accompanies the dissociation of the MAPKK–MAPK complex (Fukuda *et al.*, 1997a).

Previous studies have suggested that nuclear translocation of MAPK does not necessarily require either phosphorylation or kinase activity of MAPK, since both non-phosphorylatable MAPK mutants (Lenormand *et al.*, 1993) and a kinase-deficient mutant (Gonzalez *et al.*, 1993) were able to translocate into the nucleus normally. However, nuclear translocation of MAPK correlates with activation of the MAPK cascade. In fact, activation of MAPKK is shown to be necessary and sufficient for the nuclear translocation of MAPK (Fukuda *et al.*, 1997a; Lenormand *et al.*, 1998). Moreover, Khokhlatchev *et al.* (1998) have demonstrated that phosphorylation of MAPK induces its homodimerization and promotes nuclear translocation of MAPK. Therefore, phosphorylation of MAPK should have some role in its nuclear transport. However, we have not yet elucidated even a possible correlation between the phosphorylation state of MAPK and the dissociation of MAPK from MAPKK, a step that is a prerequisite for subsequent nuclear translocation of MAPK. Thus, the requirement of phosphorylation of MAPK for its transport has been a matter of controversy. Moreover, the mechanism by which MAPK passes through a nuclear pore has not been elucidated.

In this paper we show first that disruption of the cytoplasmic localization of endogenous MAPKK by treatment with leptomycin B, a specific inhibitor of NES-mediated nuclear export, induces nuclear translocation of MAPK, supporting our model that MAPKK acts as a cytoplasmic anchor of MAPK. Secondly, phosphorylation of MAPK by MAPKK is shown to be necessary and sufficient for the dissociation of MAPK from MAPKK. In agreement with these findings, inhibition of the phosphorylation of MAPK in the cytoplasm, but not in the nucleus, prevented the nuclear translocation of MAPK.

Moreover, phosphorylation of MAPK and its dissociation from MAPKK occurred almost simultaneously. Furthermore, a mutant MAPK that cannot dissociate from MAPKK was unable to translocate to the nucleus even after stimulation of the MAPK cascade, suggesting that dissociation of MAPK from MAPKK is necessary for the nuclear translocation of MAPK. We then show that neither wheat germ agglutinin (WGA), an inhibitor of nuclear pore-mediated active transport, nor RanQ69L, a well-known inhibitor of active nuclear transport, blocks the nuclear entry of MAPK, demonstrating that MAPK can enter the nucleus by passive diffusion. Surprisingly, however, a β -galactosidase-fused MAPK, which is too large to enter the nucleus by diffusion, was still able to enter the nucleus upon stimulation, and in this case nuclear entry was inhibited by either WGA or RanQ69L. Interestingly, when a dimerization-deficient mutant of MAPK was fused to β -galactosidase, it was unable to enter the nucleus. These results suggest that MAPK can be actively transported into the nucleus only when it is in dimer. Collectively, our results demonstrate that phosphorylation of MAPK is necessary and sufficient for its dissociation from its cytoplasmic anchor MAPKK, and that there are two mechanisms for the subsequent nuclear entry of MAPK: one is passive diffusion and the other is active transport, the latter being dependent upon the formation of a MAPK dimer.

Results

Leptomycin B treatment induces nuclear translocation of MAPK

Cytoplasmic localization of MAPKK is achieved by its NES (Fukuda *et al.*, 1996). In quiescent cells MAPK predominantly localizes to the cytoplasm and MAPK binds to the N-terminus of MAPKK (Bardwell *et al.*, 1996; Fukuda *et al.*, 1997a). We then proposed a hypothesis in which MAPKK acts as a cytoplasmic anchor of MAPK (Fukuda *et al.*, 1997a). To test this hypothesis further, leptomycin B, which has recently been shown to be a specific inhibitor of NES-mediated active nuclear export (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997b; Ossareh-Nazari *et al.*, 1997; Wolff *et al.*, 1997), was added to serum-starved *Xenopus* A6 cells, and the subcellular localization of endogenous MAPK (*Xenopus* MAPK=MPK1/ERK2) was determined. Leptomycin B disrupted the cytoplasmic localization of MAPKK (*Xenopus* MAPKK or MEK1), as demonstrated previously (Figure 1B; Fukuda *et al.*, 1997b). As shown in Figure 1A, in almost all of the cells examined (>99%) MAPK localized exclusively to the cytoplasm prior to treatment with leptomycin B. Faint nuclear staining for MAPK became apparent within 10 min of treatment, and after 30 min MAPK was distributed evenly in both the nucleus and the cytoplasm. After 2 h, strong nuclear accumulation of MAPK was detected in 10–20% of cells. Similar results were obtained in rat 3Y1 cells (data not shown). This supports our idea that MAPKK acts as a cytoplasmic anchor of MAPK.

Tyrosine phosphorylation of MAPK induces dissociation of the MAPKK–MAPK complex

Upon activation of the MAPK cascade, MAPK translocates to the nucleus, whereas MAPKK stays in the cytoplasm.

Fig. 1. Leptomycin B induces nuclear translocation of MAPK. (A) A6 cells were serum-starved for 36 h and then treated with leptomycin B (8 ng/ml). Cells were fixed at the indicated times, and subcellular localization of endogenous MAPK was detected by staining with anti-*Xenopus* MAPK antibody. Experiments were performed three times with similar results. (B) A6 cells were transfected with HA-MAPKK and serum-starved for 24 h. The cells were then treated with leptomycin B (8 ng/ml) for 2 h. Cells were fixed and then stained with anti-HA antibody.

Therefore, dissociation of the MAPKK–MAPK complex should accompany the nuclear translocation of MAPK. The concurrence of the dissociation of MAPK and its nuclear import has been demonstrated previously (Fukuda *et al.*, 1997a). We then examined the mechanisms that induce the dissociation of the complex. In this series of experiments, HA-tagged MAPKKs (*Xenopus* MAPKK or MEK1) were co-expressed with MAPKs (*Xenopus* MPK1/ERK2) in COS7 cells, and the cell lysates were subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-MAPK antibody to assess the binding between MAPKK and MAPK. MAPK co-precipitated well with wild-type MAPKK in the absence of stimulation. Co-expression of Ras Val12, which stimulates the MAPK cascade, resulted in a marked decrease in the co-precipitated MAPK (Figure 2A). Phosphorylated MAPK was dissociated completely from MAPKK and

Fig. 2. Phosphorylation of MAPK is necessary and sufficient for the dissociation of the MAPKK-MAPK complex. (A) Effects of mutations of MAPKK on the dissociation of the complex. COS7 cells were cotransfected with MAPK (*Xenopus* MAPK=MPK1/ERK2) and HA-tagged MAPKK (*Xenopus* MAPKK or MEK1) mutants with or without Ras Val12. Aliquots of whole-cell extracts were subjected to SDS-PAGE (Whole Extract) or to immunoprecipitation with anti-HA monoclonal antibody (α HA IP). Following SDS-PAGE, the samples were immunoblotted with anti-HA antibody or anti-MAPK antibody. (B) MKP-3 partially restores the association of MAPK with MAPKK. COS7 cells were cotransfected with indicated clones, and immunoprecipitations were performed as above. MKP-3 CS is a phosphatase-dead mutant. (C) Effects of mutations of MAPK on the dissociation of the complex. COS7 cells were cotransfected with the indicated clones, and immunoprecipitations were performed as in (A). (D) The kinetics of the dissociation of the MAPKK-MAPK complex. Δ B-Raf:ER cells were cotransfected with MAPK and HA-MAPKK. The cells were stimulated with 4-HT (1 μ M) for the indicated times, and immunoprecipitations were performed as above. (E) Effect of a dimerization-deficient mutation of MAPK (H181E L₄A MAPK) on its dissociation from the MAPKK-MAPK complex. COS7 cells were cotransfected with indicated clones, and immunoprecipitations were performed as in (A). The data presented in (A-E) are representatives of 2-6 independent experiments, and the total amount of transfected DNA was kept constant by addition of the appropriate empty vector.

Table I. MAPK mutants and MAPKK mutants used in this study

	Name of mutant	Mutations introduced ^a	Significance
MAPK	T188A	Thr188→Ala	one (Thr) of two activation phosphorylation sites by MAPKK is disrupted
	Y190F	Tyr190→Phe	one (Tyr) of two activation phosphorylation sites by MAPKK is disrupted
	K57R	Lys57→Arg	kinase deficient
	H181EL ₄ A	His181→Glu Leu338, 341, 346, 349→Ala	dimerization deficient
MAPKK	S218A/S222A	Ser218, 222→Ala	two activation phosphorylation sites by MAPKKK are disrupted
	K97S	Lys97→Ser	kinase deficient
	T388A	Thr388→Ala	feedback phosphorylation site by MAPK is disrupted
	SDSE	Ser218→Asp Ser222→Glu	constitutively active

^aThe numbers shown are from the sequences of *Xenopus* MAPK (MPK1/ERK2) and *Xenopus* MAPKK (MEK1).

only a fraction of unphosphorylated MAPK remained associated with MAPKK. T388A MAPKK, in which the only known feedback phosphorylation site by MAPK (Thr388) is mutated (Gotoh *et al.*, 1994; Table I), showed the same behavior as wild-type MAPKK. Thus, the feedback phosphorylation of MAPKK may not have any effect on the association or dissociation of MAPKK and MAPK. However, S218A/S222A MAPKK (in which two serine residues in the activation phosphorylation site are mutated; Table I) associated with MAPK even in the presence of Ras Val12 (Figure 2A). A kinase-deficient MAPKK (K97S MAPKK) also associated with MAPK in the presence of active Ras (data not shown). As S218A/S222A MAPKK and K97S MAPKK, which are dominant-negative forms of MAPKK, inhibited the activation of MAPK (Figure 2A, α MAPK, whole extract; data not shown), the results suggest that phosphorylation and/or activation of MAPK may be necessary for dissociation of the complex. In agreement with this, MKP-3, a dual-specific MAPK phosphatase that localizes to the cytoplasm (Muda *et al.*, 1996), partially reversed the SDSE MAPKK-induced phosphorylation of MAPK, and the resultant dephosphorylated MAPK associated with the SDSE MAPKK (Figure 2B) (SDSE MAPKK is the active form of MAPKK, in which two phosphorylation sites by MAPKKK, Ser218 and Ser222, are replaced by Asp and Glu, respectively; Table I).

To determine which is important for dissociation, the phosphorylation or kinase activity of MAPK, we expressed various mutants of MAPK together with wild-type MAPKK or the constitutively active MAPKK (SDSE MAPKK). The expressed mutant MAPKs were T188A MAPK, in which one of the two phosphorylation sites (Thr188) is mutated, Y190F MAPK, in which the other activation phosphorylation site (Tyr190) is mutated, and K57R MAPK, which is a kinase-deficient form of MAPK (Table I). Whereas all these mutant MAPKs and wild-type MAPK bound to wild-type MAPKK, T188A MAPK, K57R MAPK and wild-type MAPK, but not Y190F MAPK, dissociated from MAPKK when SDSE MAPKK was used (Figure 2C). This result suggests that phosphorylation of MAPK, especially phosphorylation of Tyr190, is necessary for the dissociation of the MAPKK–MAPK complex after stimulation, because SDSE MAPKK is able to bind to MAPK when MAPK is not phosphorylated (Figure 2B). Since these three mutant MAPKs inhibited the activation of MAPK to almost the same extent (data not shown), the activated kinase activity of MAPK is not necessary for dissociation, although the possible involvement of the weak basal kinase activity of MAPK cannot be excluded completely.

If phosphorylation of MAPK by MAPKK is necessary and sufficient for the dissociation of the MAPKK–MAPK complex, then MAPK would dissociate from MAPKK immediately after phosphorylation of MAPK. To test this, we used Δ B-Raf:ER cells, in which B-Raf can be conditionally activated by the addition of estradiol (Pritchard *et al.*, 1995). Thus, we co-expressed HA-MAPKK and MAPK in Δ B-Raf:ER cells, and added 4-hydroxy tamoxifen (4-HT) to the cultures to activate B-Raf. Maximal activation of MAPK was induced within 5 min of treatment with 4-HT and lasted for at least 2 h (Figure 2D, whole extract, α MAPK). The co-immunoprecipitation experiment showed that almost complete

dissociation of the MAPKK–MAPK complex was observed as soon as 5 min after treatment with 4-HT (Figure 2D). This result demonstrates the concurrence of the phosphorylation of MAPK and its dissociation from the complex, and therefore is consistent with our idea that phosphorylation of MAPK, probably on Tyr190, is necessary and sufficient for dissociation of the MAPKK–MAPK complex.

Dissociation of MAPK from MAPKK is necessary for nuclear translocation of MAPK

If MAPKK is a cytoplasmic anchor of MAPK, then inhibition of the dissociation of MAPK from MAPKK should lead to the inhibition of nuclear translocation of MAPK. We therefore tested the ability of several MAPK mutants to translocate into the nucleus. When wild-type MAPK was co-transfected with SDSE MAPKK in NIH 3T3 cells, MAPK strongly accumulated in the nucleus. T188A MAPK and K57R MAPK, which are partially defective in their dissociation from MAPKK, translocated to the nucleus. However, Y190F MAPK, which cannot dissociate from MAPKK, did not enter the nucleus at all (Figure 3A). These results suggest that dissociation of MAPK from MAPKK is necessary for nuclear translocation of MAPK.

To further confirm this result, we examined the effect of inhibition of the phosphorylation of endogenous MAPK on its nuclear transport. CL100 (also known as MKP-1) is a dual-specific MAPK phosphatase, and is known to localize exclusively to the nucleus (Alessi *et al.*, 1993; Brondello *et al.*, 1995). We therefore made a cDNA construct of NES–CL100, in which the NES sequence of HIV-1 Rev protein (Fischer *et al.*, 1995) was fused to the N-terminus of CL100, to localize CL100 to the cytoplasm and to inhibit the phosphorylation of MAPK in the cytoplasm. Wild-type CL100 was also used as a control. Expression of NES–CL100, but not of wild-type CL100, inhibited the dissociation of the MAPKK–MAPK complex, as did expression of MKP-3 (data not shown). As expected, when expressed in COS7 cells, NES–CL100 inhibited TPA-induced nuclear translocation of MAPK, whereas wild-type CL100 did not (Figure 3B). The inability of wild-type CL100 to inhibit MAPK nuclear translocation has been reported previously (Brondello *et al.*, 1995). These results suggest that phosphorylation of MAPK in the cytoplasm is necessary for the nuclear translocation of MAPK. Although this is consistent with our idea that phosphorylation of MAPK is required for the dissociation of MAPK from MAPKK, it is also possible that phosphorylation of MAPK has other roles in MAPK nuclear transport, such as enhancement of its dimerization (Khokhlatchev *et al.*, 1998).

Nuclear translocation of MAPK is not blocked by WGA or RanQ69L

MAPK is not a big protein (≤ 45 kDa), so it might pass through the nuclear pore simply by diffusion. However, an active transport mechanism might be involved in the process of MAPK nuclear translocation. Two known inhibitors of active nuclear transport, WGA and RanQ69L, were therefore used. WGA is known to bind to the nuclear pore complex and inhibit the nuclear localization signal (NLS)-dependent intracellular transport (Finlay *et al.*,

Fig. 3. Nuclear translocation of MAPK requires its dissociation from MAPKK. **(A)** The mutation of MAPK that prevents its dissociation from MAPKK results in the concomitant inhibition of nuclear entry of MAPK. Wild-type MAPK (WT), T188A MAPK (T188A), Y190F MAPK (Y190F), a kinase-deficient mutant of MAPK (K57R) or a dimerization-deficient mutant of MAPK (H181E L4A) was co-transfected with SDSE MAPKK (a constitutively active form of MAPKK) in NIH 3T3 cells. After 8 h of incubation, cells were fixed and stained with anti-MAPK antibody. When co-transfected with wild-type MAPKK, all MAPKs remained in the cytoplasm (data not shown). **(B)** Inhibition of phosphorylation of MAPK in the cytoplasm, but not in the nucleus, prevents the nuclear translocation of MAPK. COS7 cells were transfected with either myc-NES-CL100 or myc-CL100. Following 24 h of serum starvation, cells were left untreated (–) or stimulated with TPA (500 ng/ml) for 5 min (+). Cells were fixed and then stained with anti-myc monoclonal antibody together with anti-ERK1 polyclonal antibody. Arrowheads denote transfected cells. Experiments were performed three times with similar results.

1987; Yoneda *et al.*, 1987). RanQ69L is a GTPase-deficient mutant of Ran (Ren *et al.*, 1993; Bischoff *et al.*, 1994; Klebe *et al.*, 1995), and is reported to inhibit all known signal-dependent nuclear transports in a dominant-negative way (Dickmanns *et al.*, 1996; Palacios *et al.*, 1996; Izaurralde *et al.*, 1997). In all the injection experiments below, TRITC-BSA is co-injected with samples as a marker to denote the injected cellular compartments. When injected into the cytoplasm of A6 cells, either WGA or RanQ69L completely inhibited the nuclear translocation of FITC-NLS-BSA (i.e. NLS-dependent nuclear transport; Figure 4B, upper panels; data not shown). In contrast, neither of them blocked the serum-induced nuclear translocation of MAPK (Figure 4A and B). Therefore, an active transport mechanism may not be necessary to explain the nuclear transport mechanism of MAPK. To further address this point, the effect of WGA or RanQ69L on the kinetics of serum-induced nuclear translocation of MAPK was examined in these cells. As shown in Figure 4C, injection of WGA or RanQ69L did not delay the kinetics of nuclear translocation of MAPK; rather, they apparently accelerated the nuclear translocation of MAPK. This is probably

because WGA or RanQ69L itself is sufficient to induce nuclear translocation of MAPK to some extent in the absence of extracellular stimuli. Since WGA and RanQ69L, when injected into the cytoplasm of cells, inhibit Ran-dependent active nuclear export as well as import, they disrupt the NES-dependent cytoplasmic localization of MAPKK (data not shown). Thus, MAPK, which is retained in the cytoplasm by MAPKK, was imported concomitantly into the nucleus even in the absence of serum stimulation when WGA or RanQ69L was injected (Figure 4C; data not shown). Judging from the result shown in Figure 4C, the contribution of active transport mechanisms to the serum-induced nuclear transport of MAPK may, if anything, be small in A6 cells.

Collectively, these results suggest that MAPK has the ability to enter the nucleus normally without the aid of known active transport mechanisms.

MAPK can enter the nucleus by passive diffusion

As MAPK translocated into the nucleus in the presence of WGA or RanQ69L, it is possible that MAPK can enter the nucleus simply by passive diffusion. To confirm this,

we examined whether MAPK can enter the nucleus in the chilled condition or in the absence of ATP. Thus, we injected bacterially expressed recombinant *Xenopus* MAPK protein into the cytoplasm of chilled or ATP-depleted rat 3Y1 cells, and tested whether the injected MAPK could enter the nucleus. It has been reported previously that, when overexpressed, MAPK becomes localized to the nucleus even in the absence of stimulation (Lenormand *et al.*, 1993; Fukuda *et al.*, 1997a). As shown in Figure 5, slight but significant nuclear localization of

cytoplasmically injected MAPK was observed 1 h after injection in untreated cells. In the chilled or ATP-depleted cells, MAPK became localized to the nucleus even more significantly (Figure 5). These results indicate that MAPK has the ability to enter the nucleus by passive diffusion. Stronger nuclear localization of MAPK in the chilled or ATP-depleted cells may result from the disruption of cytoplasmic localization of MAPKK, a cytoplasmic anchor of MAPK, due to inhibition of the NES-dependent active nuclear export of MAPKK. Indeed, cooling or ATP-

Fig. 4. Nuclear import of endogenous MAPK is not blocked by WGA or by RanQ69L. (A) A6 cells were serum starved for 36 h. The cytoplasm was then injected with control buffer or WGA (2 mg/ml) together with TRITC-labeled BSA (TRITC-BSA), followed by stimulation with 10% FCS for 4 h. Cells were fixed and stained with anti-MAPK antibody. Under the same conditions, nuclear import of the NLS (SV40 NLS)-conjugated BSA (B) was inhibited completely (data not shown). (B) A6 cells were serum starved for 36 h. Control buffer or RanQ69L (9.3 mg/ml) and TRITC-BSA were then injected into the cytoplasm together with (upper panels) or without (lower panels) fluorescein isothiocyanate (FITC)-labeled NLS-BSA (FITC-NLS-BSA, 1.5 mg/ml), followed by 4 h of stimulation with FCS. Cells were fixed, and then stained with anti-MAPK antibody (lower panels) or directly subjected to observation (upper panels). The experiments in (A) and (B) were performed at least three times with similar results. (C) A6 cells were serum starved for 36 h. The cytoplasm was then injected with control buffer (●, ○), WGA (2 mg/ml, ▲, △) or RanQ69L (9.3 mg/ml, ■, □) together with TRITC-BSA. Cells were stimulated with FCS (closed symbols) or left untreated (open symbols), and incubated for 0, 1, 2 or 4 h, and then fixed and stained with anti-MAPK antibody. Quantification of the data from two independent experiments is shown. The percentages of cells in which nuclear MAPK staining was stronger than, or equal to, cytoplasmic MAPK staining are shown in each time point. Between 16 and 269 cells were examined in each time point. (D) 3Y1 cells were serum starved for 36 h. Then the cytoplasm was injected with control buffer or WGA (2 mg/ml) together with TRITC-BSA followed by stimulation with TPA (100 ng/ml) for 5 min. Cells were fixed, and then stained with anti-ERK1 antibody. Arrowheads denote injected cells. Experiments were performed twice with similar results.

depletion treatment of cells induced nuclear localization of MAPKK (data not shown).

MAPK can be actively transported into the nucleus

To further assess the possible involvement of an active transport mechanism in the nuclear transport of MAPK, we generated a cDNA construct of β -gal-MAPK, in which β -galactosidase is fused to the N-terminus of MAPK, to make MAPK too large (~160 kDa) to enter the nucleus by diffusion. To induce nuclear translocation of exogenously expressed MAPK in a stimulus-dependent manner, we used Δ B-Raf:ER cells. Δ B-Raf:ER cells were co-transfected with HA-MAPKK and β -gal-MAPK or normal MAPK, and then 4-HT was added to the cultures. Surprisingly, β -gal-MAPK was able to translocate into the nucleus

after stimulation (Figure 6B); β -gal-MAPK entered the nucleus faster and more extensively than did normal MAPK (Figure 6B and C). The difference was also observed in the presence of leptomycin B, which might inhibit the export (data not shown), being consistent with the idea that the rate of import is enhanced in β -gal-MAPK (see Discussion). This result raises the possibility that although MAPK can enter the nucleus by diffusion, it can also be transported actively.

The effect of WGA and RanQ69L on the transport of β -gal-MAPK was then tested. In this series of experiments, the nuclei of Δ B-Raf:ER cells were injected with plasmids encoding HA-tagged MAPKK and normal MAPK or β -gal-MAPK, and the cells were then incubated for 4 h to express the injected constructs. Just prior to the stimulation with 4-HT, the cytoplasms of the same cells were injected with WGA or RanQ69L. As shown in Figure 7, both WGA and RanQ69L completely blocked the nuclear import of β -gal-MAPK. Therefore, the involvement of a Ran-dependent, active transport mechanism is suggested in the nuclear translocation of MAPK. Interestingly, in this experimental system the stimulus-dependent nuclear import of overexpressed normal MAPK was also inhibited to some extent by WGA and RanQ69L (data not shown; see Discussion).

Unlike the serum-induced nuclear translocation of MAPK in Δ B-Raf:ER cells or A6 cells, which requires several hours to attain the maximal accumulation in the nucleus, the TPA-induced nuclear translocation of MAPK in 3Y1 cells occurs very rapidly, within 5 min of stimulation. Surprisingly, as shown in Figure 4D, this rapid nuclear translocation of MAPK was partially, but significantly,

Fig. 5. MAPK can enter the nucleus by passive diffusion. His-tagged MAPK protein (4.5 mg/ml) was co-injected with TRITC-BSA into the cytoplasm of 3Y1 cells, which were untreated (left), chilled (middle) or incubated in medium supplemented with 6 mM deoxyglucose and 10 mM sodium azide (right). Cells were incubated for 1 h and then fixed and stained with anti-MAPK antibody. Experiments were performed three times with similar results.

Fig. 6. MAPK dimer is actively transported into the nucleus. (A) Δ B-Raf:ER cells were co-transfected with SR α HA MAPKK and pcDNA3 MAPK (●) or pCS2 H181E L₄A MAPK (○). Cells were stimulated with 4-HT (1 μ M), incubated for 0, 1, 2, 4 or 6 h, and then fixed and stained with anti-HA and anti-MAPK antibodies. Quantification of the data from a representative experiment is shown. The percentages of cells in which nuclear MAPK staining was stronger than, or equal to, cytoplasmic MAPK staining are shown in each time point. One hundred cells were examined at each time point. The same experiments were performed twice with similar results. (B and C) Δ B-Raf:ER cells were co-transfected with SR α HA MAPKK together with pcDNA3 MAPK, pCS2 β -gal-MAPK or pCS2 β -gal-H181E L₄A MAPK. Cells were stimulated with 4-HT (1 μ M), incubated for 0, 1, 2, 4 or 6 h, and then fixed and stained with anti-HA and anti-MAPK antibodies. (B) Representative staining images of MAPKs at 0, 2 or 6 h after stimulation are shown. Experiments were performed at least three times with similar results. (C) 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 for each time point. One hundred cells were examined at each time point. Wild-type MAPK (○), β -gal-MAPK (□), β -gal-H181E L₄A MAPK (△).

Fig. 7. Nuclear import of β -gal-MAPK is blocked by WGA and RanQ69L. The nuclei of Δ B-Raf:ER cells were injected with SR α HA MAPKK together with pcDNA3 MAPK or pCS2 β -gal-MAPK (100 μ g/ml each, total 200 μ g/ml) and incubated for 4 h. The cytoplasm of the same cells were then co-injected with either WGA (2 mg/ml) or RanQ69L (14.4 mg/ml) together with TRITC-BSA, and the cells were immediately stimulated with 4-HT. After 2 h cells were fixed and then stained with anti-HA and anti-MAPK antibodies. (A) Representative images are shown. (B) Quantification of the data from three independent experiments. The percentages of cells in which nuclear MAPK staining was stronger than, or equal to, cytoplasmic MAPK staining are shown for each condition. Between 13 and 179 cells were examined for each condition in one experiment.

inhibited by WGA. Thus, in some cases the active transport mechanism may be significantly involved in the nuclear translocation of MAPK.

Taken together, these results suggest that the active transport mechanism, although not absolutely necessary (Figure 4), contributes to nuclear entry of normal MAPK (see Discussion).

Homodimerization is required for the active nuclear transport of MAPK

Khokhlatchev *et al.* (1998) recently reported that phosphorylation of MAPK induces its homodimerization to promote its nuclear translocation. In the report, they described a dimerization-deficient mutant of ERK2 MAPK, H176E L₄A ERK2, which corresponds to H181E L₄A MAPK in *Xenopus* MPK1/ERK2 (Table I). Thus, we examined the behavior of this mutant MAPK in our assay systems. H181E L₄A MAPK dissociated from MAPKK after Ras activation to the same extent as wild-type MAPK (Figure 2E). Dimerization of MAPK is therefore not a necessary step for the dissociation of the MAPKK–MAPK complex. Moreover, co-expression of SDSE MAPKK, but not of wild-type MAPKK (data not shown), induced

nuclear accumulation of H181E L₄A MAPK in NIH 3T3 cells (Figure 3A). These results suggest that homodimerization of MAPK is not necessarily required for its nuclear translocation.

However, because co-expression of SDSE MAPKK was adopted as a method to activate the MAPK cascade in the experiment described above, the time course could not be measured. To examine in more detail the effect of homodimerization of MAPK on its nuclear transport, we followed the kinetics of nuclear translocation of H181E L₄A MAPK in Δ B-Raf:ER cells. In this system H181E L₄A MAPK also translocated into the nucleus upon stimulation, but did so more slowly than wild-type MAPK (Figure 6A), although the time course and the extent of phosphorylation were almost the same in both wild-type MAPK and H181E L₄A MAPK (data not shown). Wild-type MAPK entered the nucleus more quickly than H181E L₄A MAPK even in the presence of leptomycin B, although the nuclear entry of both forms of MAPK was enhanced because the nuclear translocation of MAPKK, which is a cytoplasmic anchor of MAPK, was induced by the drug treatment (data not shown). Thus, the difference observed may arise from a change in the rate of import. Therefore, dimerization may have a role in promoting the nuclear translocation of MAPK.

We then tested whether β -gal-fused H181E L₄A MAPK could be translocated to the nucleus in Δ B-Raf:ER cells. Interestingly, β -gal–H181E L₄A MAPK did not enter the nucleus at all (Figure 6B and C). It is suggested, therefore, that active nuclear transport of MAPK is dependent on the formation of MAPK dimer.

Discussion

MAPKK is a cytoplasmic anchor of MAPK

In this study we addressed the mechanisms of nuclear translocation of MAPK. First, we reinforced our idea that MAPKK acts as a cytoplasmic anchor of MAPK, by demonstrating that disruption of cytoplasmic localization of MAPKK by leptomycin B leads to concomitant nuclear localization of MAPK. Therefore, dissociation of the MAPKK–MAPK complex should be an initial, primary step in the nuclear translocation of MAPK. In agreement with this, Y190F MAPK, which is unable to dissociate from MAPKK even after stimulation of MAPKK (see below), could not translocate into the nucleus at all. Therefore, dissociation of MAPK from MAPKK is necessary for the nuclear translocation of MAPK. It should be noted that disruption of the cytoplasmic localization of MAPKK by either leptomycin B treatment or introduction of the mutation in the NES of MAPKK (Leu to Ala) did not lead to dissociation of the MAPKK–MAPK complex (data not shown). Thus, it is likely that in these cases MAPK interacts with MAPKK in the nucleus, after both MAPK and MAPKK pass through the nuclear pore as monomers. Their nuclear translocation is possible because the association between MAPK and MAPKK is reversible.

Dissociation of the MAPKK–MAPK complex by tyrosine phosphorylation of MAPK

We have then shown that phosphorylation of MAPK, especially on Tyr190, is necessary and sufficient for the dissociation of the complex. Inhibition of the tyrosine

phosphorylation of MAPK by phenylalanine substitution or phosphatase treatment resulted in inhibition of the dissociation of MAPK from MAPKK. Moreover, phosphorylation of MAPK and dissociation of the MAPKK–MAPK complex occurred almost simultaneously. The kinase activity of MAPK may be unnecessary for the dissociation because the kinase-deficient form of MAPK can be dissociated from MAPKK after phosphorylation. Although a trace amount of phosphorylated T188A MAPK or phosphorylated K57R MAPK co-precipitated with MAPKK in some cases (Figure 2C), this would be explained as a non-specific binding probably caused by the mutation of MAPK. In fact, we always observed the complete dissociation of wild-type MAPK from MAPKK after phosphorylation of MAPK in a whole series of experiments performed in this study. Although we cannot rule out completely the involvement of the basal, weak activity of MAPK in the dissociation of the complex, it is clear from the present experiments that the feedback phosphorylation of MAPKK on Thr388 by MAPK is not involved in the dissociation of the complex. MAPKK contains a possible candidate of phosphorylation by MAPK *in vitro* within the MAPK binding site (Thr23), our experiment showed that replacement of Thr23 by Ala had no effect on the formation and dissociation of the MAPKK–MAPK complex (data not shown). Moreover, MP1, which was recently reported to promote binding between MAPKK and MAPK (only ERK1), does not possess any consensus motif of MAPK phosphorylation (Schaeffer *et al.*, 1998). Furthermore, concurrence of the phosphorylation of MAPK by MAPKK and dissociation of the MAPKK–MAPK complex may also be compatible with the idea that the downstream events of MAPK activation are not involved in the dissociation of the complex. Therefore, there is no evidence for the involvement of the kinase activity of MAPK in the formation and dissociation of the MAPKK–MAPK complex.

T188A MAPK was slightly more resistant than wild-type MAPK to the dissociation after stimulation, although the data in Figure 2C did not show this clearly. Therefore, it is possible that phosphorylation of Thr188 enhances the dissociation of MAPK from MAPKK, which is primarily induced by Tyr190 phosphorylation. It may be worth mentioning the previous work of Haystead *et al.* (1992) reporting that Tyr phosphorylation precedes Thr phosphorylation. Anyway, it is possible that the conformational change of MAPK caused by its phosphorylation is responsible for dissociation of the complex (Canagarajah *et al.*, 1997).

Phosphorylation state of MAPK and its nuclear translocation

Lenormand *et al.* (1993) previously suggested that phosphorylation of MAPK is not necessary for its nuclear translocation, based on their result that TA- or YF-mutants expressed in cultured cells translocated to the nucleus, as did wild-type MAPK, which appears to be different from our results. However, in their experiment mutant MAPKs were overexpressed, so the expressed MAPKs were no longer retained to the cytoplasmic anchor, MAPKK. Therefore, the process of the dissociation of MAPK from MAPKK, an initial and prerequisite step for MAPK nuclear translocation under normal conditions, was bypassed under

their experimental conditions. Moreover, the stimulus-dependent accumulation of MAPK they observed may be accounted for by their recent observation indicating the stimulus-dependent synthesis of the nuclear anchor of MAPK (Lenormand *et al.*, 1998). Therefore, it is possible that only phosphorylated MAPK can enter the nucleus. In contrast, Khokhlatchev *et al.* (1998) demonstrated that phosphorylated MAPK can form dimers with either phosphorylated or unphosphorylated MAPK to promote their nuclear localization. Therefore, if phosphorylated MAPK were to compete with MAPKK for unphosphorylated MAPK, dissociation of unphosphorylated MAPK from MAPKK could be stimulated by phosphorylation of endogenous MAPK. This mechanism might account for part of the result obtained by Lenormand *et al.* (1993), and may suggest the possibility that even unphosphorylated MAPK dissociates from MAPKK after phosphorylation of part of MAPK and enters the nucleus. This mechanism should be tested in future studies.

Although phosphorylation of MAPK peaks as early as 5–10 min after stimulation in most cases, nuclear accumulation of MAPK is generally slow; it takes 4–6 h to attain maximal accumulation in some cases. As the dissociation of MAPK from MAPKK and/or the dimer formation may be coupled to the phosphorylation of MAPK, they are not a rate-limiting step. Lenormand *et al.* (1998) suggested the existence of an as yet unidentified nuclear anchor(s), whose synthesis requires activation of the MAPK pathway. Thus, even when MAPK is phosphorylated and transported into the nucleus following the stimulation of cells, it would be exported immediately from the nucleus unless the nuclear anchor is synthesized sufficiently.

Passive diffusion and active transport of MAPK

Our experiments examining the effects of RanQ69L and WGA on the nuclear transport of endogenous MAPK suggest that MAPK can enter the nucleus simply by diffusion. However, we have also shown that β -gal-MAPK, which is too large to pass through the nuclear pore by diffusion, could still enter the nucleus upon stimulation and that the nuclear transport of β -gal-MAPK is dependent on the formation of MAPK dimer and mediated by a WGA-sensitive, Ran-dependent mechanism. We therefore propose that MAPK is translocated into the nucleus through two distinct mechanisms, passive diffusion and active transport.

Why two mechanisms exist for nuclear import of MAPK is unclear. As shown in Figure 4C, passive diffusion may be sufficient to explain the serum-induced nuclear transport of MAPK in A6 cells, which requires several hours to achieve the maximal nuclear accumulation. However, as shown in Figure 4D, TPA-induced nuclear translocation of MAPK in 3Y1 cells, whose maximal level is attained within 5 min of stimulation, was partially but significantly inhibited by WGA, suggesting the involvement of an active transport mechanism. Therefore, it is possible that the active transport mechanism is required for rapid nuclear translocation of MAPK if it occurs.

We do not know, however, how the dimerization-dependent, Ran-mediated active transport of MAPK is achieved. Because Ran is known to be involved in the NLS-mediated transport mechanism (for review see Nigg,

Fig. 8. A hypothetical model for nuclear translocation of MAPK. See text for details.

1997), two possibilities may be apparent; one is that the dimer formation produces an NLS, which is not present in monomeric MAPK molecule, and the other is that the MAPK dimer can associate with other protein(s) which possesses an NLS.

Nuclear import of overexpressed MAPK, unlike that of endogenous MAPK, was significantly inhibited by WGA or RanQ69L (data not shown). It is possible that overexpression of MAPK would increase the concentration of MAPK in the cytoplasm and consequently the probability of dimers forming. Therefore, the contribution of a dimerization-dependent active transport mechanism may increase in cells overexpressing MAPK. Indeed, the speed of nuclear import of overexpressed wild-type MAPK, which can be potentially transported by both passive diffusion and active transport mechanisms, was significantly higher than that of overexpressed dimerization-deficient mutant of MAPK, which can be transported only by passive diffusion (Figure 6A). That nuclear import of β -gal-MAPK is faster and more extensive than that of normal MAPK (Figure 6B and C) may be explained in a similar way. Thus, because β -galactosidase has the ability to form tetramers, β -gal-MAPK may be more likely to form dimers than normal MAPK.

Conclusions

In summary, we have approached the mechanisms of nuclear transport of classical MAPK and described a scenario for the outlines of these processes (Figure 8). MAPK localizes to the cytoplasm by binding to the cytoplasmic anchor MAPKK, a direct activator of MAPK, under quiescent conditions. After stimulation, MAPK

becomes phosphorylated by MAPKK and dissociates from MAPKK. The MAPKK-mediated phosphorylation of MAPK, especially the tyrosine phosphorylation, is necessary and sufficient for the dissociation of MAPK from MAPKK. The dissociated MAPK then enters the nucleus by either passive diffusion or active transport mechanisms. The nuclear accumulation of MAPK may require a putative nuclear anchor(s). Interestingly, this outline of nuclear import of MAPK is similar, but not identical, to that of fission yeast stress-activated MAPK Spc1, which was demonstrated recently by Gaits *et al.* (1998). They have shown that nuclear translocation of Spc1 is coupled with dissociation from its activator kinase Wis1, although Wis1 does not seem to act as a cytoplasmic anchor of Spc1, and that Spc1 must be phosphorylated by Wis1 to localize in the nucleus and nuclear retention of Spc1 requires a nuclear anchor. Thus, the mechanism of nuclear localization of MAPK may be evolutionarily conserved in some aspects. Future studies are required to elucidate the mechanisms for nuclear translocation of other families of the MAPK superfamily such as JNK/SAPK, p38 and ERK5/BMK1 in higher eukaryotes.

Materials and methods

Cell culture and microinjection

Xenopus A6 cells were cultured in Leibovitz's L-15 medium supplemented with 10% fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 0.2 mg/ml kanamycin). Δ B-Raf:ER cells, COS7 cells and rat 3Y1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics as above. 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), except for RanQ69L (see below). For the chilling studies, 3Y1 cells were cooled on ice for 30 min prior to injection, and injection was performed on a cooled microscope stage. For ATP-depletion studies, 3Y1 cells were incubated 30 min prior to, during, and 1 h following injection in glucose-free DMEM supplemented with 6 mM deoxyglucose and 10 mM sodium azide.

Cell staining

Cells were fixed by the direct addition of 3.7% formaldehyde (final concentration) to the cell culture medium and then permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. The coverslips were incubated with 3% bovine serum albumin (BSA)/PBS dissolving 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), a mouse monoclonal anti-HA antibody (12CA5, 1:200) and a mouse monoclonal antibody to myc epitope (9E10 Santa Cruz, 1:100). Cells were finally mounted in Mowiol and examined using a Zeiss Axiophoto.

Transfections and immunoprecipitation

Immunoprecipitation assays were performed as described previously (Fukuda *et al.*, 1997a), with some modifications. COS7 or Δ B-Raf:ER cells were seeded in 60 mm dishes 16 h before transfection at a density of 6×10^5 or 3.6×10^5 cells per dish, respectively. In cotransfection, Ras Val12, HA-MAPKKs and MAPKs were introduced at a ratio of 2:5:5 (Figure 2A and E), HA-SDSE MAPKK, MAPK and MKP-3 were at a ratio of 1:1:4 (Figure 2B), HA-SDSE MAPKK and MAPKs were at a ratio of 1:1 (Figure 2C) and HA-MAPKK and MAPK were at a ratio of 1:1 (Figure 2D). The total amount of transfected DNA was 6 μ g (COS7 cells) or 3.6 μ g (Δ B-Raf:ER cells) per dish. The plasmids were mixed with LipofectAMINE (Life Technologies, Inc.; COS7 cells) or with LipofectAMINE PLUS (Life Technologies, Inc.) and LipofectAMINE (Δ B-Raf:ER cells) in serum-free Opti-MEM (Life Technologies, Inc.) and the cells were transfected. Either 5 h (COS7 cells) or 2 h (Δ B-Raf:ER cells) after transfection, the cells were incubated in DMEM supplemented with 10% FCS for an additional 24 h and

further incubated in serum-free DMEM for 12 h (COS7 cells) or incubated in DMEM supplemented with 10% FCS for 36 h (Δ B-Raf:ER cells) before extraction. Cells were washed with ice-cold extraction buffer (E-buffer; 20 mM HEPES–NaOH, pH 7.4, 2 mM MgCl₂, 2 mM EGTA), and scraped into 300 μ l of E-buffer containing 1% aprotinin, 1 mM PhCH₂SO₂F, 2 mM dithiothreitol (DTT), 1 mM Na₃VO₄ and 1 mM NaF (E*-buffer). Following homogenization, the cell lysates were centrifuged at 15 000 g for 30 min. A portion (240 μ l) of the supernatant was then mixed and incubated with 7.5 μ l of anti-HA antibody (12CA5; 5 mg/ml) and 50 μ l of protein A–Sepharose beads (Pharmacia, Uppsala, Sweden) at 4°C for 1.5 h. The beads were washed four times with 300 μ l of E*-buffer by inverting tubes several times, and eluted by incubation with 300 μ l of an elution buffer (100 mM Gly–HCl, pH 2.2) for 1 min, a portion (150 μ l) of the eluate was then mixed immediately with 12 μ l of a neutralization buffer (1.5 M Tris–HCl, pH 8.8). The eluted samples were subjected to immunoblotting with anti-HA antibody and anti-*Xenopus* MAPK antibody.

Transfection into NIH 3T3 cells and Δ B-Raf:ER cells for cell staining experiments were performed using LipofectAMINE and LipofectAMINE PLUS reagent according to the manufacturer's instructions with the use of 1.2 μ g of total DNA per 35 mm dish.

DNA construction

The open reading frames (ORF) of T388A and K97S *Xenopus* MAPKK (Gotoh *et al.*, 1994) were amplified by PCR with a 5' primer 5'-ACTCAGATCTAACATGCCTAAAAAGAAG-3' and a 3' primer 5'-GCCAAGATCTCTCACTCCGGCGGCAT-3' (generating *Bgl*III sites at both ends of the MAPKK). The mutagenesis of Ser218 and Ser222 to Ala was performed using the Quickchange site-directed mutagenesis kit (Stratagene) with the mutagenic primers 5'-GGG-CAACTCATAGACGCCATGGCAAATGCCTTTGTTGGGACAAGA-TCC-3' and 5'-GGATCTTGTCCCAACAAAGGCATTGGCCATGG-CGTCTATGAGTTGCCC-3' using wild-type MAPKK amplified with the above primers and subcloned into pCR-Blunt (Invitrogen) as a template. Each *Bgl*III fragment was cloned into pSR α HA1, yielding pSR α HA-T388A MAPKK, pSR α HA-K97S MAPKK and pSR α HA-S218A/S222A MAPKK, respectively. pMT-SM MKP-3 myc, pMT-SM MKP-3 (C293S) myc and empty vector pMT-SM were kindly provided by Dr S.Arkininstall. The ORF of wild-type *Xenopus* MAPK was amplified by PCR with a 5' primer 5'-CCGGGATCCCATGGCAGCGGAG-GAGCTGCGTCT-3' and a 3' primer 5'-GGCCGAATTCTCAGTA-CCCTGGCTGGAATCTAGC-3' (generating *Bam*HI site and *Eco*RI site at the 5' and 3' ends, respectively) and subcloned into pCR-Blunt, and used as a template for generating K57R MAPK with the mutagenic primers 5'-CGAGTTGCTATCAGGAAAATCAGCCC-3' and 5'-GGG-CTGATTTTCCTGATAGCAACTCG-3', and H181E L₄A MAPK with the mutagenic primers 5'-GCAGACCCAGATGAGGATCACACTGGC-3' and 5'-GCCAGTGTGATCTCATCTGGGTCTGC-3' (His181 to Glu), 5'-TTTGAATGGAGGCTGATGATGCGCCCAAGGAGACTC-3' and 5'-GAGTCTCCTTGGGCGCATCATCAGCCTCCATTTCAA-3' (Leu 338, 341 to Ala) and 5'-GCGCCCAAGGAGACTGCTAAGGA-GGCAATTTTGAAGAAACC-3' and 5'-GGTTTCTCAAAAATTG-CCTCCTTAGCAGTCTCCTTGGGCGC-3' (Leu 346, 349 to Ala). The *Bam*HI fragments (containing vector sequence at its 3' end) was subcloned into pSR α 456, yielding pSR α K57R MAPK and H181E L₄A MAPK. A *Bam*HI fragment of wild-type MAPK was subcloned into the *Bam*HI site of pcDNA3, to yield pcDNA3 MAPK. Replacement of Tyr188 by Ala or that of Thr190 by Phe was performed using the method described by Kunkel *et al.* (1987) with the mutagenic primers 5'-ACTGGCTTTCTCGCAGAATATGTAG-3' for pSR α T188A MAPK and 5'-TTCTCACAGAATTTGTAGCCACTCG-3' for pSR α Y190F MAPK, respectively. To obtain pSR α myc CL100, a *Bam*HI fragment of CL100 cDNA was subcloned into pSR α myc1. Myc epitope tag amplified by PCR was introduced into pSR α 456, yielding pSR α -myc2. pSR α myc2-NES1 was obtained as described before (Fukuda *et al.*, 1997c), using pSR α -myc2 and the synthetic oligonucleotides 5'-GATCCTCTTC-AGCTACCAACCGCTTGAGAGACTTACTCTTGATTGTAGATCTG-3' and 5'-AATTCAAGATCTACAACTCAAGAGTAAGTCTCTCAAGCGG-TGGTAGCTGAAGAGG-3' (introduced *Bgl*III site underlined). A *Bam*HI fragment containing CL100 was cloned into pSR α -myc2-NES1, yielding pSR α -myc NES-CL100. cDNA encoding Ran was isolated from Quick-Clone cDNA (Clontech) by PCR with 5' primer 5'-ATGGCTGCGCAG-GGAGAGCCCC-3' and 3' primer 5'-TCACAGGTCATCATCCTCAT-CCGGGAGAGC-3' and cloned into pCR-Blunt. The mutation of Gln69 to Leu was performed using the mutagenic primers 5'-GACACAGCGG-GCCTGGAGAAATTC-3' and 5'-GAATTTCTCCAGGCGGCTGTG-TCT-3'. An *Eco*RI fragment (containing vector sequences at both ends)

was inserted into the *Eco*RI site of pGEX-6P1 to obtain pGEX-6P1 RanQ69L. The ORF of the *Escherichia coli* β -galactosidase gene was amplified by PCR with 5' primer 5'-CCGGGATCCATGATTACGGAT-TCACTGGCCGTCGTTTAC-3' and 3' primer 5'-GGGAGATCTTTT-TTGACACCAAGCAACTGGTAATGGTAGCG-3' (generating *Bam*HI and *Bgl*III sites at 5' and 3' ends of β -galactosidase, respectively), and the *Bam*HI–*Bgl*III fragment was subcloned into the *Bgl*III site of pCS4-HA to obtain pCS4-HA β -gal. The ORF of *Xenopus* MAPK was amplified using 5' primer 5'-GCCGGGATCCCATGGCAGCGGAGG-3' and 3' primer (described above), and the *Bam*HI–*Eco*RI fragment was subcloned into the *Bam*HI–*Eco*RI site of pCS2, yielding pCS2 MAPK. pCS2 MAPK was digested with *Bam*HI and the resulting fragment was ligated with *Bam*HI–*Bgl*III fragment of β -galactosidase to yield pCS2 β -gal-MAPK. pSR α H181E L₄A MAPK was digested with *Bgl*III and *Xho*I, and the resulting fragment containing the C-terminal half of H181E L₄A MAPK was cloned into pCS2 MAPK and pCS2 β -gal-MAPK which were digested with *Bgl*III and *Xho*I, to obtain pCS2 H181E L₄A MAPK and pCS2 β -gal-H181E L₄A MAPK, respectively.

Preparation of recombinant protein

His-tagged *Xenopus* MAPK protein was prepared as described previously (Gotoh *et al.*, 1994). RanQ69L was expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* BL21 using pGEX-6P1 constructs. Bacteria were grown in Luria–Bertani medium at 37°C to an optical density of 0.6, and expression was induced for 6 h at 25°C by the addition of 0.5 mM isopropyl- β -D-galactoside (IPTG). Cells were harvested by centrifugation for 10 min at 6000 g and resuspended on ice in a lysis buffer (200 mM K₂HPO₄/KH₂PO₄–KOH, pH 7.0, 50 mM NaCl, 1 mM EGTA, 1 mM DTT, 0.1% aprotinin, 1 μ g/ml pepstatin A, 0.5 μ g/ml leupeptin). Following sonication, the lysate was centrifuged at 15 000 g for 30 min. The supernatant was mixed and incubated with glutathione–Sepharose 4B (Pharmacia) equilibrated with the lysis buffer. After washing, the beads were further incubated with the lysis buffer (without protease inhibitors) containing PreScission Protease (Pharmacia) to cleave GST from the fusion protein. The cleaved protein in lysis buffer was incubated in 20 mM EDTA with 500 μ M GTP γ S for 2 h on ice, then dialyzed against Ran injection buffer (20 mM HEPES–KOH, pH 7.4, 120 mM KCl, 1 mM MgCl₂). Resultant RanQ69L–GTP γ S was concentrated using Microcon-10 (Amicon). Aliquots were frozen in liquid nitrogen and stored at –80°C.

Acknowledgements

We thank Dr S.Arkininstall for kindly providing us with expression constructs of MKP-3, Dr S.M.Keyse for cDNA of CL100, Dr M.Yoshida for leptomycin B and Dr M.McMahon for Δ B-Raf:ER cells. We also thank S.Asano for construction of pGEX-6P1 RanQ69L and members of our laboratory for their helpful comments and suggestions. This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan (to E.N.).

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Received April 12, 1999; revised July 20, 1999;
accepted August 3, 1999