Evidence for Existence of a Nuclear Pore Complex-mediated, Cytosol-independent Pathway of Nuclear Translocation of ERK MAP Kinase in Permeabilized Cells*

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The classical mitogen-activated protein kinase (MAPK, also known as ERK) pathway is widely involved in eukaryotic signal transductions. In response to extracellular stimuli, MAPK becomes activated and translocates from the cytoplasm to the nucleus. At least two pathways for the nuclear import of MAPK are shown to exist; passive diffusion of a monomer and Ran-depend-ent active transport of a dimer, the detailed molecular mechanism of which is unknown. In this study, we have reconstituted nuclear import of MAPK in vitro by using digitonin-permeabilized cells with GFP-fused MAPK protein (GFP-MAPK), which is too large to pass through the nuclear pore by passive diffusion. GFP-MAPK was able to accumulate in the nucleus irrespective of its phosphorylation state. This import of GFP-MAPK occurred even in the absence of any soluble cytosolic factors or ATP but was inhibited by wheat germ agglutinin or an excess amount of importin-β or at low temperatures. Moreover, MAPK directly bound to an FG repeat region of nucleoporin CAN/Nup214 in vitro. Taken together, these results suggest the third pathway for nuclear import of MAPK, in which MAPK passes through the nuclear pore by directly interacting with the nuclear pore complex.

The classical mitogen-activated protein kinase (MAPK, also known as ERK) cascade is among the key signaling pathways regulating many cellular events, such as cell proliferation, cell differentiation, and early embryonic development. Extracellular stimuli such as growth factors induce sequential activation of three protein kinases, MAP kinase kinase kinase; GST, glutathione S-transferase; GSH, reduced glutathione; GFP, green fluorescent protein; GTPyS, guanosine 5'-O-(thiotriphosphate); NLS, nuclear localization signal; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; NPC, nuclear pore complex; 4HT, 4-hydroxytamoxifen; WGA, wheat germ agglutinin; HA, hemagglutinin.

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EXPERIMENTAL PROCEDURES

Cell Cultures and Transfection—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum and antibiotics (100 units/ml penicillin and 0.2 mg/ml kanamycin). B-Raf-ER cells (38), a gift from Dr. M. McMahon, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics. Transfection into HeLa cells and B-Raf-ER cells was performed using LipofectAMINE and LipofectAMINE PLUS reagents (Life Technologies, Inc.) according to the manufacturer’s instructions with the use of 1 μg of total DNA/35 mm dish. In cotransfection, GFP-MAPK (0.1 μg) and MAPKK (0.9 μg) were used.

DNA Construction—The mammalian expression plasmid harboring Xenopus MAPKK (i.e. MEK) (18) and the bacterial expression plasmids harboring His-tagged Xenopus MAPK (17), His-tagged kinase-dead Xenopus MAPK (39), His-tagged constitutively active Xenopus MAPKK (LA-SDSE, see Ref. 40), and GST-RanQ69L (19) were constructed previously. To obtain a mammalian expression plasmid harboring GFP-MAPK, the open reading frame of Xenopus MAPK (i.e. ERK2) was
cloned into pEGFP-C3 vector (CLONTECH). This GFP-MAPK plasmid and pEGFP-C1 vector were polymerase chain reaction with a 5′ primer, 5′-AAAGCTTCACTGCTGACAAAGGCGGAGG-3′, and a 3′ primer, 5′-GAATTCGTCGACATCTTCTACATGTCG-3′, generating XhoI sites at both ends. Each XhoI fragment was cloned into pEGFP-C3 (CLONTECH) for expression. Site-directed mutagenesis was performed on phosphokinase domain for expression of GFP-MAPK in Escherichia coli. To construct a bacterial expression plasmid for GST-CAN(FG) a region corresponding to amino acid residues 1691–1894 of human CAN/Nup214 was amplified from Quick clone HeLa cDNA (CLONTECH) by polymerase chain reaction with a 5′ primer, 5′-GAATTCGTCGACATCTTCTACATGTCG-3′, and a 3′ primer, 5′-ACGAGATCCGATGACTTCTAGACCTCG-3′, generating BglII and BamHI ends. The BglII-BamHI fragment was cloned into the BamHI site of pGEX-6p-3 (Amersham Pharmacia Biotech). Into the resultant GST-GFP vector, the open reading frame of wild-type, K57R, or TAYF-Xenopus MAPK (19) was cloned. The prokaryotic expression vector for HA-tagged importin-β was a gift from Dr. Y. Yoneda (Osaka University, Osaka, Japan).

**Purification of Bacterially Expressed Recombinant Proteins**—The purification and expression of His-GFP, His-GFP-MAPK, and His-MAPK were performed according to the method described previously (20) with some modifications. Proteins were expressed and purified on ProBond Resin (Invitrogen) and Q-Sepharose (Amersham Pharmacia Biotech). The accumulation rate in small aliquots. All the recombinant proteins used in this study were purified from the soluble fraction of E. coli lysates.

**Conjugation of a Fluorescent Probe and a Nuclear Localization Signal (NLS) Peptide with BSA**—Fluorescein isothiocyanate (FITC, Sigma) (250 μl, 1 mg/ml in Me2SO) was mixed with BSA (Sigma) (5 ml, 4 mg/ml in 0.1M sodium carbonate, pH 9.0) and incubated overnight at 4°C. NH4Cl (final 50 mM) was then added. After gel filtration, the resultant FITC-BSA was dialyzed against phosphate-buffered saline, pH 8.0. FITC-BSA was conjugated with a synthetic peptide containing the NLS of SV40 large T-antigen (CPKKRVEDP) using the bifunctional cross-linker sulfo-SMCC (Pierce).

**Phosphorylation of Recombinant MAPKs**—Active Xenopus MAPK was purified from Xenopus egg extracts by sequential chromatography on Q-Sepharose, S-Sepharose, and Hitrap Heparin (Amersham Pharmacia Biotech) (43) and incubated with GFP-MAPK in a buffer of 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 0.1 mM EDTA, 2 mM dithiothreitol, 1 mM vanadate, 12.5 mM β-glycerophosphate, 1 μg/ml each of aprotinin, leupeptin, and pepstatin for 3 h at 37°C. The sample was then dialyzed against buffer A and concentrated. The SDS-polyacrylamide gel electrophoresis analysis showed that approximately half of the MAPK was converted to the phosphorylated activated form, and the rest of MAPK was ~15% of that of MAPK.

**Phosphorylation of Cells and Import Assays**—We slightly modified a protocol of Adam et al. (21, 44). HeLa cells were plated on glass coverslips 2 days before use. After washing in ice-cold buffer D (buffer B containing 2 mM dithiothreitol, 1 mM vanadate, 12.5 mM β-glycerophosphate, 1 μg/ml each of aprotinin, leupeptin, and pepstatin), cells were permeabilized with digitonin (60 μg/ml, Calbiochem) in buffer D for 5 min on ice. Cells were then washed with buffer D. A standard import assay consisted of the import substrate (GFP fusion proteins or FITC-NLS-BSA), Xenopus oocyte cytosol (3 mg/ml, prepared as described previously (21) and dialyzed against buffer D) and an ATP-regenerating system (final 1 mM ATP, 5 mM creatine phosphate (Sigma), 20 units/ml creatine phosphokinase (Nacalai)) in buffer D. The final concentrations of the import substrates were 0.3–1.0 μM (GFP and GFP-MAPK) and 30–60 μg/ml (FITC-NLS-BSA). The final concentration of kinase-dead MAPK or LA-SDSE MAPK was 2 μM, that of GST-RanQ69L-GTP·S·P was 3 mg/ml, and that of importin-β was 0.5 mg/ml. Unless otherwise stated, fluorescence was observed without fixation. To fix the permeabilized cells, the samples were incubated in 4% paraformaldehyde/2% sucrose/phosphate-buffered saline for 1 h at 25°C and then incubated in methanol/acetone (50%/50%) for 30 min at –20°C. A perfusion chamber was made according to Lombillo et al. (45). After cell permeabilization, two facing edges of a coverslip were sealed with vaseline, and the coverslip was inverted over a drop of buffer D on a microscope slide. Import substrates were perfused into this chamber, and time lapse imaging of nuclear import was carried out. Microscopy was performed with a Zeiss Axiohot2, a Photometrics CCD camera, and software IP Lab Spectrum (Scanalytics, Inc). Images were processed using IPLab Spectrum or Adobe Photoshop (Adobe Systems). Quantification of fluorescence was performed using IP Lab Spectrum.

**Relative Accumulation Rate of Import Substrates**—A field was chosen at random before the perfusion of import substrates. An import substrate was then perfused into the chamber, and time lapse imaging of nuclear import was carried out. Microscopy was performed with a Zeiss Axiohot2, a Photometrics CCD camera, and software IP Lab Spectrum (Scanalytics, Inc). Images were processed using IPLab Spectrum or Adobe Photoshop (Adobe Systems). Quantification of fluorescence was performed using IP Lab Spectrum.

**In Vitro Binding Assay—Lysates from bacteria expressing GST or GST-CAN(FG) were incubated with GSH-Sepharose 4B (Amersham Pharmacia Biotech) and washed with buffer C. Bound proteins were eluted with 10 mM GSH/buffer C. EDTA (final 0.1 mM) and TGT·PS·S (final 0.2 mM) were added to eluted proteins, and the solution was incubated overnight on ice. Resultant GST-RanQ69L-GTP·S·P was dialyzed against buffer B and concentrated using Centricron 30 (Amicon). Recombinant mouse importin-β was prepared as described previously (42). Proteins were frozen in liquid nitrogen and stored at −80°C in small aliquots. All the recombinant proteins used in this study were purified from the soluble fraction of E. coli lysates.

**RESULTS**

**Nuclear Transport of GFP-MAPK in Permeabilized Cells**—To analyze the mechanism of nuclear translocation of ERK MAPK in more detail, we used the digitoquinin-permeabilized cell system with GFP-fused Xenopus ERK2 MAPK (GFP-MAPK) as import substrates. The complete system consisted of digitoquinin-permeabilized HeLa cells, Xenopus oocyte extracts (called “cytosol” hereafter), and the ATP-regenerating system (called “ATP” hereafter). In this assay system, we can follow the behaviors of import substrates without fixation.

We performed experiments using His-tagged GFP and His-tagged GFP-MAPK as import substrates in the complete assay system. When GFP was used, it entered the nucleus slowly and eventually became distributed equally inside and outside the nucleus (Fig. 1, A and B, GFP). In contrast, GFP-MAPK first accumulated at the nuclear rim (Fig. 1A, GFP-MAPK, arrows heads) and then became concentrated in the nuclei rapidly (Fig. 1, A and B, GFP-MAPK). The accumulation rate of GFP-MAPK at the nuclear rim implies the interaction of GFP-MAPK with the nuclear pore complex (NPC). As GFP did not accumulate at the nuclear rim (Fig. 1A, upper), the MAPK portion of GFP-MAPK may interact with the NPC. Phosphorylation of MAPK by MEK...
MAPKK did not affect significantly the mode and kinetics of nuclear translocation of GFP-MAPK (Fig. 1A, GFP-MAPK (Phospho)). Thus, GFP-MAPK was able to translocate to the nucleus irrespective of its phosphorylation state. These observations, together with the fact that the molecular mass of GFP-MAPK (~70 kDa) is above the diffusion limit of the nuclear pore, suggest that non-phosphorylated MAPK is able to pass through the nuclear pore by a non-diffusion mechanism.

We examined the behavior of GFP-MAPK in living cells. When expressed in HeLa cells, GFP-MAPK localized to both the nucleus and the cytoplasm. The concentration in the nucleus was often higher than in the cytoplasm (Fig. 1C, left, -MAPKK). When MEK-MAPKK was coexpressed, GFP-MAPK was excluded from the nucleus (Fig. 1C, left, +MAPKK). These observations suggest that GFP-MAPK is also retained to the cytoplasm by MAPKK, and that free GFP-MAPK can enter the nucleus like non-tag MAPK, despite the larger size of GFP-MAPK. We next expressed both GFP-MAPK and MAPKK in ΔB-Raf-ER cells, in which B-Raf can be activated conditionally by the addition of estradiol or 4-hydroxytamoxifen (4HT) (38). Upon the addition of 4HT, GFP-MAPK translocated from the cytoplasm to the nucleus (Fig. 1C, right two panels). This finding suggests that GFP-MAPK, like non-tag MAPK, dissociates from MAPKK upon phosphorylation by MAPKK, and dissociated GFP-MAPK translocates to the nucleus. All these characteristics of intracellular dynamics of GFP-MAPK are apparently not distinguishable from those of non-tag wild-type MAPK.

**Characteristics of the Nuclear Import of GFP-MAPK—MEK MAPKK acts as a cytoplasmic anchor of MAPK, and phosphorylation of MAPK by activated MAPKK results in the dissociation of MAPK from MAPKK (18, 19). We examined the effect of the addition of MEK-MAPKK on nuclear translocation of GFP-MAPK in the complete in vitro system. Although kinase-dead MAPKK suppressed the nuclear accumulation of GFP-MAPK (Fig. 2A, Kinase Dead), constitutively active MAPKK did not (Fig. 2A, Active). Therefore, MAPKK can act as a cytoplasmic anchor of MAPK in this in vitro system.

Wheat germ agglutinin (WGA) binds to glycosylated nucleoporins and blocks the NPC-mediated transport (46, 47). We examined the effect of WGA on the nuclear translocation of GFP-MAPK in permeabilized cells. In cells treated with WGA, the translocation of GFP-MAPK was markedly delayed (Fig. 2B). The nuclear import of GFP-phosphorylated MAPK was also delayed by WGA (data not shown). These results suggest the involvement of the NPC in the nuclear translocation of GFP-MAPK.

We then examined the effect of RanQ69L on the nuclear translocation of GFP-MAPK. We used FITC-BSA, which was conjugated to the NLS peptide of SV40 large T-antigen (FITC-NLS-BSA) as a control import substrate. FITC-NLS-BSA (i.e., NLS-BSA) translocated and accumulated to the nucleus efficiently in our permeabilized cell system (Fig. 2, C and D, NLS-BSA and Cont.). This nuclear import of NLS-BSA was almost completely inhibited in the presence of GST-RanQ69L-GFP-S (Fig. 2, C and D, NLS-BSA and RanQ69L). In contrast, the nuclear import of GFP-MAPK was not inhibited at all under the same conditions (Fig. 2, C and D, GFP-MAPK and RanQ69L). Moreover, whereas NLS-BSA was unable to translocate to the nucleus in the absence of cytosol or ATP, the nuclear translocation of GFP-MAPK occurred as efficiently as in the presence of cytosol and ATP, i.e. in the complete assay system (Fig. 2, C and D, -Cytosol). These results suggest that GFP-MAPK can translocate into the nucleus without cytosolic factors or ATP. The nuclear import of GFP-MAPK in permeabilized cells in the absence of cytosol or ATP was suppressed by WGA by the presence of an excess amount of importin-β or at low temperatures (Fig. 2E). These results indicate that the nuclear translocation of GFP-MAPK is not a diffusion process but involves the NPC. It has previously been reported that NPC-mediated transport of proteins such as β-catenin and RCC1 that is different from the conventional NLS-dependent transport is also blocked by an excess amount of importin-β (48–50).

Taken together, our results suggest the existence of a hitherto unidentified pathway for nuclear import of MAPK in which MAPK passes through the nuclear pore by interacting directly with the NPC irrespective of the phosphorylation state of MAPK. Our experiments with another GFP-MAPK (called “non-His GFP-MAPK” hereafter) that was produced by cleav-

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**Fig. 1.** Nuclear translocation of GFP-MAPK in vitro (A) or in living cells (C). A, time lapse imaging of nuclear translocation of GFP (top), GFP-MAPK (middle), and GFP-phospho MAPK (bottom) in the complete assay system in vitro. The arrows indicate the nuclei where GFP-MAPK or GFP-phospho MAPK accumulated at the rim. B, quantification of the data from representative experiments. Relative nuclear accumulation rate R of GFP or GFP-MAPK was calculated for each nucleus in the field. R = 0 means that the fluorescence within the nucleus is equal to background (for details, see under “Experimental Procedures.” Mean R of all the nuclei was plotted against time (n = 26 for GFP and n = 18 for GFP-MAPK). C, left two panels, GFP-MAPK was expressed in HeLa cells with an empty vector (−MAPKK) or with MAPKK (+MAPKK). Right two panels, GFP-MAPK and MAPKK were coexpressed in ΔB-Raf-ER cells. −4HT, control; +4HT, 2 h after addition of 4HT (1 μM). Scale bar = 50 μm in A; scale bar = 20 μm in C.
ing GST-GFP-MAPK produced essentially the same results. This non-His GFP-MAPK does not have His tag unlike the previous GFP-MAPK used above, and amino acid residues between GFP and MAPK are different. Thus, non-His GFP-MAPK translocated into the nuclei of permeabilized cells in the absence of cytosol or ATP efficiently (data not shown), confirming our results. Moreover, non-His GFP-TAYF MAPK, which are the kinase-dead mutant and the unphosphorylatable mutant, respectively, translocated into the nuclei as efficiently as did non-His GFP-MAPK (data not shown). These results confirm that neither the kinase activity of MAPK nor the phosphorylation of MAPK by MEK is required for nuclear translocation of GFP-MAPK in this in vitro system.

**Direct Binding of MAPK to an FG Repeat Region of Nucleoporin**

We then examined the interaction between MAPK and an FG repeat region of CAN/Nup214 in vitro. Nucleoporins containing an FG repeat motif are thought to be common docking sites for those proteins that pass through the NPC, such as importin-β and β-catenin (for review, see Ref. 51). We constructed a fusion protein between GST and an FG repeat-containing fragment of human nucleoporin CAN/Nup214 (called GST-CAN(FG)). This fragment has been shown to interact with importin-β family proteins (52). In control experiments, HA-importin-β bound to GST-CAN(FG) but did not bind to GST (Fig. 3, lower). GFP-MAPK but not GFP specifically bound to GST-CAN(FG) (Fig. 3, upper). GFP-MAPK, which had been phosphorylated by MEK, also bound specifically to GST-CAN(FG) (Fig. 3, upper). In addition, His-tagged wild-type MAPK (His-MAPK) also bound to GST-CAN(FG) (Fig. 3, lower). These results suggest that MAPK is able to bind directly to the FG repeat region of CAN/Nup214, supporting the idea that MAPK can be imported into the nucleus through direct interaction with the NPC.

**GFP-MAPK Can Be Exported from the Nucleus**

We examined whether GFP-MAPK is exported from the nucleus. We first incubated GFP-MAPK with permeabilized cells to translocate it to the nucleus. Then, the cells were washed and incubated with the buffer solution, and the behavior of nuclear GFP-MAPK was followed. To see the effect of photobleaching of GFP-MAPK, the fluorescence of fixed GFP-MAPK was also...
followed. The fluorescence of unfixed GFP-MAPK in the nuclei faded remarkably with time, whereas that of fixed GFP-MAPK was not reduced significantly (Fig. 4, A and B). These results indicate that GFP-MAPK is able to be exported from the nucleus. Because the above experiments were performed in the absence of cytosol or ATP, the export of GFP-MAPK does not seem to be mediated by the MAPKK-dependent mechanism we reported previously (53).

**DISCUSSION**

In this study, we succeeded in reconstituting the nuclear transport of MAPK using GFP-MAPK and the digitonin-permeabilized cell system. Our experiments identified a novel pathway for nuclear translocation of MAPK, i.e. MAPK can be imported into the nucleus through direct interaction with the NPC (Fig. 4C). This novel pathway for nuclear import of MAPK is distinct from passive diffusion of a MAPK monomer or active transport of a dimer. GFP-MAPK (~70 kDa) is too large to pass through the nuclear pore by passive diffusion, and our data indicate that GFP-MAPK is imported into the nucleus by an NPC-mediated but cytosol- or ATP-independent mechanism. Moreover, MAPK is able to interact with an FG repeat-containing fragment of nucleoporin CAN/Nup214, suggesting that MAPK binds to the NPC, although it is not known whether CAN/Nup214 is an in vivo docking site for MAPK. Thus, MAPK seems to dock directly to the nuclear pore complex and pass through the nuclear pore by interacting with nucleoporins. This conclusion is in good agreement with the observation that the overexpression of GFP-MAPK results in its nuclear accumulation (Fig. 1C and Ref. 55).

It has been demonstrated that not only MAPK but also several other proteins are able to translocate to the nucleus independently of cytosolic factors. Those proteins include importin-β, HIV-1 Vpr, β-catenin, and RCC1 (42, 48–50, 56). It will be of interest as to whether these proteins use the same mechanism to pass through the nuclear pore. The fact that the import of GFP-MAPK was inhibited by importin-β suggests that MAPK binds to the same nucleoporins as does importin-β, or the access of MAPK to the NPC is sterically blocked by importin-β. The identification of MAPK-binding proteins in the NPC is among the key questions to be elucidated. In any case, this mechanism may be identical or similar to the so-called facilitated diffusion mechanism that requires specific, low affinity interactions with nucleoporins (37, 50, 57, 58). The result that GFP-MAPK can be exported from the nucleus is consistent with the model of facilitated diffusion. This sort of nuclear translocation occurs under conditions of minimal energy and is believed to be propelled by Brownian motion, which is dependent on temperature.

Although facilitated diffusion cannot occur against a concentration gradient, GFP-MAPK is able to become concentrated in the nucleus in our experiments. It is probable that GFP-MAPK is anchored in the nucleus. Concerning the nuclear anchor for MAPK, it could be worth mentioning the previous work of Lenormand et al. (54). They have reported that the nuclear translocation of MAPK requires neosynthesis of short-lived proteins, serving as nuclear anchors for MAPK. The identification of nuclear anchor proteins would be of great help to elucidate the mechanism of nuclear accumulation of MAPK.

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