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Nuclear Export of ERK3 by a CRM1-dependent Mechanism Regulates Its Inhibitory Action on Cell Cycle Progression*

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Extracellular signal-regulated kinase 3 (ERK3) is an atypical member of the mitogen-activated protein kinase family of serine/threonine kinases. Little is known on the regulation of ERK3 function. Here, we report that ERK3 is constitutively localized in the cytoplasmic and nuclear compartments. In contrast to other mitogen-activated protein kinases, the cellular distribution of ERK3 remains unchanged in response to common mitogenic or stress stimuli and is independent of the enzymatic activity or phosphorylation of the kinase. The cytoplasmic localization of ERK3 is directed by a CRM1-dependent nuclear export mechanism. Treatment of cells with leptomycin B causes the nuclear accumulation of ERK3 in a high percentage of cells. Moreover, ectopic expression of CRM1 promotes the cytoplasmic relocalization of ERK3, whereas overexpression of CRM1-binding protein 1, which binds CRM1 with high affinity, inhibits the nuclear export of ERK3. We also show that CRM1 binds to ERK3 in vitro. Importantly, we show that enforced localization of ERK3 in the nucleus or cytoplasm markedly attenuates the ability of the kinase to induce cell cycle arrest in fibroblasts. Our results suggest that nucleocytoplasmic shuttling of ERK3 is required for its negative regulatory effect on cell cycle progression.

Recent studies have highlighted the importance of the nucleocytoplasmic transport of signaling proteins in the regulation of eukaryotic cellular responses such as cell cycle progression and cell proliferation, differentiation, stress responses, and circadian clocks (1). Small regulatory proteins can, in principle, enter and leave the nucleus by simple diffusion through the nuclear envelope to the other by a family of soluble transport receptors, classified as importins or exportins, which belong to the importin-β family of RanGTP-binding proteins. Importins bind their cargo in the cytoplasm and release it upon binding to RanGTP in the nucleus; on the opposite, exportins form a trimeric complex with cargo and RanGTP in the nucleus and release their transport substrate into the cytoplasm upon GTP hydrolysis (for review, see Refs. 2–4). At steady state, the subcellular localization of proteins is determined by the balance between import and export rates. Cargo proteins interact directly with their cognate transport receptor through specific nuclear localization signal (NLS)3 or nuclear export signal (NES) sequences or indirectly via adaptor molecules (2, 4).

MAP kinases are a family of evolutionarily conserved protein kinases that play a critical role in transducing extracellular chemical and physical signals into intracellular responses (for review, see Refs. 5–7). Upon activation, these enzymes phosphorylate a variety of substrates that are present in all compartments of the cell. Consequently, the spatial distribution of MAP kinases, as well as the temporal regulation of their activity, is an important determinant of the ultimate biological response. Studies on the regulation of MAP kinase localization have been focused mainly on the mammalian enzymes ERK1 and ERK2 (8, 9). In resting cells, unphosphorylated ERK1/2 are anchored in the cytoplasm by their association with the MAP kinase kinases MEK1/2 (10) as well as with other cellular proteins such as protein phosphatase PTP-SL (11) and the death effector domain-containing protein PEA-15 (12). After mitogenic stimulation, the MEK-ERK complex is disrupted, and a pool of ERK1/2 is translocated to the nucleus (13–15). Recent work has shown that ERK2 mainly enters the nucleus by a saturable, carrier-independent facilitated mechanism (16). It has been proposed that sustained retention of ERK1/2 in the nucleus requires the neosynthesis of nuclear anchoring proteins (17). Dephosphorylation of ERK1/2 by nuclear phosphatases promotes their binding to NES-containing MEK1/2, which then mediate the export of MAP kinases to the cytoplasm by an active CRM1-dependent mechanism (18). Studies in yeast and mammalian cells suggest that localization of MAP kinases is regulated mainly by changes in the abundance and affinity of anchoring proteins.

Several studies have emphasized the importance of the subcellular localization of MAP kinases in modulating cellular responses. Disruption of the NES was found to potentiate dramatically the ability of activated mutants of MAP kinase kinase to induce morphological changes and malignant transformation of rodent fibroblasts (19). These effects were correlated with a strong increase in the fraction of nuclear activated ERK1/2 and were reversed by coexpression of MAP kinase phosphatase-1. Ectopic expression of an activated variant of ERK2 in the nucleus was also shown to transform NIH 3T3

3 The abbreviations used are: NLS, nuclear localization signal; BrdUrd, 5-bromo-2-deoxyuridine; DAPI, 4,6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; JNK, c-Jun N-terminal kinase; LMB, leptomycin B; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NES, nuclear export signal; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate.

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cells and to induce neurite extension in PC12 cells (20). Reciprocally, sequestration of ERK1/2 in the cytoplasm by forced expression of a catalytically inactive MAP kinase phosphatase-3 mutant inhibits Elk1-dependent gene transcription and prevents S phase entry in fibroblasts (21). In astrocytes, the increased cell proliferation observed upon inactivation of the PEA-15 gene is associated with constitutive nuclear localization of ERK1/2 and augmented c-fos mRNA expression (12).

ERK3 defines a distinct subfamily of MAP kinases which also includes the related kinase p63\(^{MAPK}\) (22). ERK3 is a 100-kDa protein that shares about 50% identity with ERK1/2 in the kinase domain but presents structural features that distinguish it from conventional MAP kinases (23–25). Notably, ERK3 contains the sequence SEG instead of the conserved TXY motif in the activation loop and possesses a long C-terminal extension. Little is known about the regulation and functions of ERK3. Interestingly, we have observed recently that overexpression of ERK3 in NIH 3T3 cells markedly inhibits DNA synthesis (26). Because of the importance of MAP kinase localization in the control of their activity, we have examined the subcellular distribution and nucleocytoplasmic transport of ERK3.

Fig. 1. ERK3 is localized into the cytoplasmic and nuclear compartments. A, the indicated cell lines were transiently transfected with Myc-tagged ERK3. Exponentially proliferating cells were stained with anti-Myc antibody, and the localization of ectopic ERK3 was analyzed by fluorescence microscopy. B, localization of endogenous ERK3. Proliferating HeLa and HEK293 cells were stained with polyclonal anti-ERK3 antibody E3-CTE4 and analyzed by fluorescence microscopy. C, specificity of anti-ERK3 antibody. NIH 3T3 fibroblasts were incubated in the absence of primary antibody (− antibody), with anti-ERK3 antibody E3-CTE4, or with anti-ERK3 antibody immunodepleted on His\(^{6}\)-ERK3-GST-immobilized beads or empty beads. Staining of ERK3 was detected by immunofluorescence microscopy.

Fig. 2. Subcellular localization of ERK3 is not affected by common MAP kinase stimuli. NIH 3T3 fibroblasts were transfected with Myc-tagged ERK3 and serum-starved for 24 h (quiescent). The cells were then stimulated for 1 h with 10% serum, 50 ng/ml epidermal growth factor (EGF), 10 \(\mu\)M lysophosphatidic acid (LPA), 0.5 mM sodium arsenite, or 600 \(\mu\)M H\(_2\)O\(_2\). The localization of ectopically expressed ERK3 was analyzed by fluorescence microscopy after staining with anti-Myc antibody.
Mean 150 cells were scored for each coverslip. The nantly nuclear (Nlysine (Sigma) at a density of 40,000 cells/well of 6-well plates. Aftercrowding was generated, and then incubated with Cell Signaling Technology by immunizing rabbits with a synthetic phosphoserine 189 peptide coupled to keyhole limpet hemocyanin (26).

Plasmid Constructs and Mutagenesis—All recombinant ERK3 DNA constructs were derived from the human cDNA sequence (25). Mutations were introduced into ERK3 cDNA using the Altered Sites in vitro mutagenesis system (Promega). pcDNA3-Myc–ERK3 was constructed by inserting the Myc epitope sequence derived from pCS3MT (provided by A. J. Waskiewicz, Fred Hutchinson Cancer Research Center) into the HindIII/EcoRI sites of pcDNA3 (Invitrogen). pcDNA3-Myc–ERK3 was constructed by first inserting an EcoRI site at the initiator ATG codon of ERK3 cDNA by site-directed mutagenesis, followed by subcloning of the ERK3 coding sequence into the EcoRI site of pcDNA3-Myc–ERK3. The K49A/K50A (kinase-dead) and Ser-189 mutants of ERK3 were obtained by site-directed mutagenesis and subcloned into pcDNA3-Myc vector. The ERK3Δ, ERK3ΔΔ, and ERK3ΔΔΔ deletion mutants were generated by PCR using pcDNA3-HA–ERK3 as template and subcloned into the EcoRI/HindIII sites of pcDNA3-Myc vector. To construct the Rev–ERK3–GFP fusion plasmids, the ERK3 regions of interest were amplified by PCR and subcloned into the AgeI site of pRevL1.4–GFP (28). To generate NLS–ERK3, two annealed oligonucleotides coding for the SV40 NLS were subcloned into the NcoI/EcoRI sites of pCS3MT. The sequence PKKKKRKRL replaced the sixth Myc epitope of the Myc vector. The NES–ERK3 was engineered similarly by subcloning two annealed oligonucleotides coding for the NES (LQKKLLRLELG) into the EcoRI sites of pCS3MT. Then, the coding sequence of ERK3 was ligated in-frame into the EcoRI site of the modified pCS3MT vectors. The NES–ERK3 was obtained by subcloning oligonucleotides coding for the SV40 NLS into the EcoRI/HindIII sites of pcDNA3-Myc vector followed by ligation of the ERK3 sequence into the EcoRI site of the vector. To generate the NES–ERK3-NLS construct, oligonucleotides coding for the SV40 NLS were first subcloned into the EcoRI/HindIII sites of pCS3MT-NES followed by insertion of ERK3 coding sequence into the EcoRI site of the vector to yield pCS3MT-NES–ERK3-NLS. All mutations were confirmed by DNA sequencing. pcDNA3 vector encoding Myc-tagged CRM1 was kindly provided by C. Dargemont (Institut Jacques Monod, Paris). To construct the pcDNA3-HA–snurportin 1 expression vector, the snurportin 1 tagging sequence was inserted upstream of the Myc epitope of the Myc vector.

Preparation of Recombinant Proteins—The recombinant His6–CRM1, His6–GST, and His6–ERK3–GST proteins were expressed in Escherichia coli and purified by affinity chromatography on nickel–nitrilotriacetic acid resin (Qiagen) as described (31). His-tagged canine RanQ69L was kindly provided by D. Görlich (Universität Heidelberg) and purified as described (32).

Immunofluorescence Microscopy—Cells grown on glass coverslips were washed twice with ice-cold PBS, and then fixed with 3.7% paraformaldehyde and PBS for 20 min at 37 °C. After quenching in 0.1 M glycine and PBS for 5 min, the cells were permeabilized by incubation in 0.1% Triton X-100 and PBS for 5 min at room temperature and then blocked with 1% bovine serum albumin and PBS overnight at 4 °C. For detection of ectopically expressed ERK3, the cells were stained for 1 h at 37 °C with anti-Myc primary antibody followed by incubation with FITC-conjugated anti-mouse IgG. Transfected HA-tagged snurportin 1 was visualized using a polyclonal anti-HA primary antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody. For localization of endogenous ERK3, the cells were stained with a polyclonal anti-ERK3 antibody.

CRM1 Binding Assay—In vitro CRM1 binding assays were performed as described (33) with some modifications. The recombinant proteins His8–GST or His6–ERK3–GST (3–10 μg) were bound to glutathione–Sepharose beads (Amersham Biosciences) preblocked for 1 h
FIG. 4. **CRM1 promotes the nuclear export of ERK3.** **A,** NIH 3T3 cells were transiently transfected with 1 μg of Myc-tagged ERK3 in combination with 1 μg of Myc-CRM1 or empty vector as indicated. After 48 h, the cellular localization of ERK3 and CRM1 was analyzed by fluorescence microscopy after staining with anti-ERK3 and anti-CRM1 antibodies, respectively (top panel). The localization of ERK3 was analyzed quantitatively as described in Fig. 3 (bottom panel). The bar graph represents the mean ± S.E. of two independent experiments. **B,** NIH 3T3 cells were transiently transfected with 1 μg of Myc-tagged ERK3 along with 1 μg of HA-snurportin 1 or empty vector as indicated. The localization of ectopically expressed ERK3 and snurportin 1 was analyzed by fluorescence microscopy after staining with anti-Myc and anti-HA antibodies, respectively.

with 1% bovine serum albumin. The beads were then washed with CRM1 binding buffer (50 mM Hepes, pH 7.9, 200 mM KCl, 5 mM MgCl2, 2 mM β-mercaptoethanol, 0.4% Tween 20, 0.4% nonfat dry milk, 1 mM GTP) and used as affinity matrices. His6-CRM1 was either expressed in *E. coli* or translated *in vitro* from pET15b-CRM1 in the presence of [35S]methionine using the TNT T7 Coupled Wheat Germ Extract System (Promega) and incubated with the beads for 3 h at 4°C in a total volume of 388 μl of binding buffer. Where indicated, bacterially expressed His-tagged RanQ69L was added at a final concentration of 0.4 μM. The beads were recovered by mild centrifugation and washed four times with binding buffer. Bound proteins were resolved by SDS-PAGE and visualized by autoradiography or immunoblotting with anti-CRM1 antibody. The total amount of His6-GST or His6-ERK3-GST proteins was monitored by immunoblotting with anti-GST antibody.

**BrdUrd Incorporation Studies**—For analysis of BrdUrd incorporation, NIH 3T3 fibroblasts were seeded at 40,000 cells/well onto poly-L-lysine-coated glass coverslips placed in 6-well plates and transfected with expression plasmids (2 μg of DNA) as described above. Exponentially growing cells were then incubated for 16 h with 10 μM BrdUrd. The cells were fixed with 3.7% paraformaldehyde and PBS for 20 min at 37°C. After quenching in 0.1 M glycine for 5 min, the cells were permeabilized by incubation in 0.1% Triton X-100 for 5 min at room temperature. Nonspecific sites were blocked in 1% bovine serum albumin and PBS for 60 min at 37°C. The transfected proteins were detected by staining with anti-Myc as primary antibody and FITC-conjugated anti-rabbit IgG as secondary reagent. Then, DNA was denatured with 2 N HCl for 10 min at room temperature. Staining for BrdUrd incorporation was performed by incubating cells for 1 h at 37°C with anti-BrdUrd antibody followed by incubation with rhodamine-conjugated anti-mouse IgG as secondary reagent. The nuclei were visualized by DAPI (1 μM final concentration) staining. Cell samples were analyzed by fluorescence microscopy. The results are expressed as the percentage of transfected cells showing nuclear labeling for BrdUrd.

**RESULTS**

**Localization of ERK3 in the Cytoplasmic and Nuclear Compartments**—To determine the subcellular distribution of ERK3, we transiently expressed a Myc-tagged construct of ERK3 in various cell lines and analyzed its localization by immunofluorescence microscopy with 9E10 antibody. In the cell lines tested, Myc-ERK3 was distributed evenly in the cytoplasmic and nuclear compartments, with the exception of HeLa cells where the kinase was enriched in the nucleus (Fig. 1A). To verify that localization of ectopically expressed ERK3 reflects that of the endogenous protein, we examined the distribution of endogenous ERK3 in cells using a polyclonal anti-ERK3 antibody. As shown in Fig. 1, B and C, ERK3 staining was detected in both the cytoplasm and nucleus of exponentially proliferating cells. Preincubation of the antiserum with His6-ERK3-GST fusion protein immobilized to nickel-nitrilotriacetic acid beads, but not with empty beads, almost completely abolished the fluorescence signal, confirming the specificity of the antibody (Fig. 1C). No reactivity was detected in the absence of primary antibody.

Stimulation of cells with mitogenic factors causes the translocation of ERK1/2 MAP kinases from the cytoplasm to the nucleus (13–15). To determine whether the cellular localization of ERK3 was regulated by extracellular stimuli, NIH 3T3 cells were transfected with Myc-ERK3 and exposed to different growth factors or chemical stresses (Fig. 2). In quiescent cells, ERK3 was localized in both the cytoplasmic and nuclear compartments, whereas ERK2 was present exclusively in the cytoplasm. Stimulation with serum growth factors, epidermal
growth factor, or the G protein-coupled receptor agonist lysophosphatidic acid for 5 min to 3 h did not modify the subcellular distribution of ERK3. Under the same conditions, ERK2 was quantitatively relocalized to the nucleus (data not shown). Similarly, exposure to the oxidant H2O2 or to arsenite had no detectable effect on ERK3 localization. These experiments indicate that the cellular localization of ERK3 is not influenced by common MAP kinase stimuli.

ERK3 Is Actively Exported to the Cytoplasm by a CRM1-dependent Mechanism—Treatment of cells with the protein synthesis inhibitor cycloheximide does not modify the cellular distribution of ERK3, suggesting that the cytoplasmic localization of the kinase is ensured by a nuclear export mechanism (data not shown). To test the possibility that ERK3 is actively exported from the nucleus by the receptor CRM1 (also known as exportin 1) (34–36), we treated cells with the antibiotic LMB, a specific inhibitor of CRM1-dependent transport (37). Addition of LMB to exponentially proliferating NIH 3T3 cells caused the progressive accumulation of ERK3 in the nucleus (Fig. 3, A and B). Quantification of the results revealed that ERK3 staining was primarily nuclear in less than 5% of control cells, whereas ~60% of cells displayed predominant nuclear staining after 12 h of treatment with LMB (Fig. 3B). To establish further the role of CRM1 in ERK3 transport, we tested the effect of CRM1 overexpression on the cellular localization of the kinase. As shown in Fig. 4A, ectopic expression of CRM1 promoted the cytoplasmic relocalization of ERK3 in 40% of cotransfected cells, again suggesting that CRM1 directs ERK3 nuclear export.

Snurportin 1 is an adaptor protein that mediates nuclear import of m3G-capped uridine-rich snRNPs by the importin-β receptor pathway (29). After each round of import, snurportin 1 must return to the cytoplasm, and this recycling is mediated by the export receptor CRM1 (30). Notably, snurportin 1 binds CRM1 with a much higher affinity than the leucine-rich NES-containing protein Rev and competes with CRM1-dependent RNA export pathways in vivo. From this, it has been suggested that sensitivity toward competition by snurportin 1 would represent a stringent test for CRM1 requirement (30). We thus exploited this idea to test further the involvement of CRM1 in mediating ERK3 export. When snurportin 1 was coexpressed with ERK3 in NIH 3T3 cells, the localization of ERK3 became predominantly nuclear (Fig. 4B). Quantification of the results indicated that around 60% of cells cotransfected with snurportin 1 display enriched nuclear staining for ERK3, in contrast to less than 5% of control cells.

We used an in vitro binding assay to determine whether CRM1 interacts directly with ERK3. CRM1 was translated in vitro with a wheat germ extract (to eliminate the contribution of endogenous lysate proteins) and incubated with the indicated Myc-tagged ERK3 constructs. The localization of ectopically expressed ERK3 was determined by fluorescence microscopy after staining with anti-Myc antibody. Quantitative analysis of the localization data is presented in the bar graph, which represents the mean ± S.E. of two independent experiments.

CRM1-mediated Nuclear Export of ERK3

Fig. 5. ERK3 interacts with CRM1 in vitro. A, CRM1 was translated in vitro from a wheat germ extract in the presence of [35S]methionine and incubated for 3 h with His8-GST or His8-ERK3-GST fusion proteins bound to glutathione-agarose beads. Where indicated, 0.4 μM RanQ69L was added to the reaction. Bound proteins were analyzed by SDS-PAGE followed by fluorography. The lower panels show the Coomassie staining of the starting material. B, recombinant His8-CRM1 purified from E. coli was incubated with His8-GST or His8-ERK3-GST fusion proteins as above. Bound material was analyzed by immunoblotting with anti-CRM1 antibody.

Fig. 6. Nuclear export of ERK3 is independent of activation loop phosphorylation or kinase activity. NIH 3T3 cells were transiently transfected with the indicated Myc-tagged ERK3 constructs. The localization of ectopically expressed ERK3 was determined by fluorescence microscopy after staining with anti-Myc antibody. Quantitative analysis of the localization data is presented in the bar graph, which represents the mean ± S.E. of two independent experiments.
bound to His6-ERK3-GST, confirming that the interaction between the two proteins is direct (Fig. 5B). Altogether, our results demonstrate that ERK3 is actively exported to the cytoplasm by the exportin CRM1.

Activation Loop Phosphorylation and Kinase Activity of ERK3 Are Not Required for Its Nuclear Export—It has been proposed that ERK1/2 dephosphorylation is required for its nuclear export by MEK (18). To determine whether activation loop phosphorylation and kinase activity of ERK3 influence its cellular distribution, we examined the behavior of various ERK3 mutants. The two lysine residues present in subdomain II of ERK3 were mutated to alanine to generate a kinase-dead enzyme, and the activation loop Ser-189 was replaced by either alanine (S189A) or by a phosphomimetic aspartate (S189D). ERK3 mutants. The two lysine residues present in subdomain II of ERK3 were mutated to alanine to generate a kinase-dead enzyme, and the activation loop Ser-189 was replaced by either alanine (S189A) or by a phosphomimetic aspartate (S189D). ERK3 has been shown to be phosphorylated on Ser189 alanine (S189A) or by a phosphomimetic aspartate (S189D) residue. ERK3 has been shown to be phosphorylated on Ser189 in vivo (26, 38). No change in the cellular localization of ERK3 was observed for any of these mutants (Fig. 6), suggesting that nuclear export of ERK3 is independent of enzymatic activation and Ser-189 phosphorylation/dephosphorylation. To substantiate this, we examined the localization of phosphorylated ERK3 using a phosphoserine 189-specific antibody. In proliferating NIH 3T3 fibroblasts, the Ser-189-phosphorylated form of ERK3 was distributed evenly between the cytoplasm and nucleus (Fig. 7). Treatment of cells with LMB led to a redistribution of phosphorylated ERK3 to the nuclear compartment. The immunostaining pattern of Ser-189-phosphorylated ERK3 was superimposable to that of total ERK3, consistent with the idea that activation loop phosphorylation does not regulate ERK3 transport.

Mapping the Regions of ERK3 Confering Nuclear Export Activity—CRM1 has been identified as the receptor for leucine-rich NES proteins (2, 4). This type of export signal is characterized by the presence of four critically spaced hydrophobic residues, most often represented by leucine (39). ERK3 possesses several motifs that resemble the leucine-rich NES (Table I). To address the contribution of these sequences to the nuclear export of ERK3, we mutated two of the characteristic hydrophobic residues within eight different NES-like motifs and analyzed the cellular localization of the resulting mutants. Immunofluorescence analysis revealed that all of the individual ERK3 mutants have a cellular distribution similar to that of the wild-type protein (Table I), indicating that either none of these motifs has export activity or that mutation of a single motif is not sufficient to disrupt CRM1-mediated export.

To identify the export signal of ERK3, we also generated a series of truncations of the C-terminal extension of the kinase (Fig. 8A). Deletion of the last 179 amino acids of ERK3 had no significant impact on its cellular localization (Fig. 8B). However, further deletion of the region comprised between amino acids 399 and 542 (ERK3(399–542)) resulted in the nuclear accumulation of the protein in ~25% of transfected cells. The smaller ERK3(366–542) mutant had the same cellular distribution as the 1–398 deletion mutant. These results suggest that a functional export signal may reside within residues 399–542 of ERK3. However, we cannot exclude the possibility that deletion of residues 399–542 accelerates the nuclear import of ERK3. To distinguish between these possibilities, we tested the intrinsic export activity of different regions of ERK3, encompassing amino acids 399–542, using the Rev-GFP nuclear export assay (28). The ERK3 region comprising amino acids 297–542 displayed an export activity comparable with that of IκB NES in this assay (Fig. 8C). Removal of amino acids 297–365 or 366–542 abolished the export activity, indicating that either the two fragments interact synergistically to form a functional export sequence or that the global conformation of the ERK3(297–542) region is important for receptor binding and nuclear export. We conclude from these data that residues 297–542 of ERK3 contain nuclear export activity.

ERK3 Is Imported in the Nucleus by a Temperature-dependent Mechanism—The molecular mass of ERK3 (~100 kDa) suggests that the protein enters the nucleus by an active transport mechanism. To address this point further, we tested the temperature dependence of ERK3 nuclear import. Exponentially proliferating NIH 3T3 cells transfected with Myc-ERK3 were treated with LMB at either 37 or 4 °C, and the localization of the protein was monitored by immunofluorescence. As shown in Fig. 9, ERK3 accumulated in the nucleus of 25% of cells after 1 h of exposure to LMB at 37 °C. However, the nuclear accumulation of ERK3 was completely abrogated at low temperature, implying that ERK3 is imported by an active mechanism.

ERK3 does not contain a classical single or bipartite NLS. However, a lysine-rich sequence is found between residues 489 and 494 of human ERK3 (25). To test whether this region constitutes an authentic NLS, we examined the subcellular

**Table I**

<table>
<thead>
<tr>
<th>Sequence mutated</th>
<th>Cells with N = C</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>0.0 ± 2.3</td>
</tr>
<tr>
<td>EDEVDDILLMD (345)</td>
<td>0.0 ± 2.9</td>
</tr>
<tr>
<td>FDSIAGTGL (339)</td>
<td>91.2 ± 2.8</td>
</tr>
<tr>
<td>VSQLLEKL (568)</td>
<td>96.1 ± 0.6</td>
</tr>
<tr>
<td>LFNQ (568)</td>
<td>94.2 ± 3.0</td>
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The localization of ectopically expressed ERK3 was determined by fluorescence microscopy after staining with anti-Myc antibody. The percentage of cells with a uniform distribution of ERK3 between the cytoplasm and nucleus (N = C) was scored. Results represents the mean ± S. E. of two experiments. Hydrophobic amino acids are in bold, and residues mutated to alanine are underlined.
localization of a mutant ERK3 protein with alanine substitutions of lysine residues 489, 490, and 492. Mutation of these basic amino acids had no detectable influence on the cellular distribution of ERK3 (data not shown), suggesting that an adaptor molecule or a distinct class of signal mediates the nuclear import of the kinase.

Nucleocytoplasmic Shuttling of ERK3 Is Necessary for Its Inhibitory Effect on DNA Synthesis—We have shown recently that overexpression of ERK3 inhibits S phase entry in NIH 3T3 fibroblasts (26). To determine whether the subcellular localization of ERK3 regulates its ability to inhibit cell cycle progression, we introduced by mutagenesis a strong NLS (from SV40) or NES (from MEK1) at the N terminus or C terminus of ERK3 to force its expression in the nuclear or cytoplasmic compartment. As predicted, addition of the SV40 NLS to ERK3 was sufficient to induce its nuclear accumulation in a high proportion of cells, whereas the NES-ERK3 fusion protein localized predominantly to the cytoplasm (Fig. 10A). To evaluate the impact of these ERK3 constructs on the cell cycle, exponentially proliferating NIH 3T3 cells were incubated for 16 h with BrdUrd to monitor S phase entry (Fig. 10, B and C). Ectopic expression of p27Kip1, used as positive control, markedly decreased the number of BrdUrd-positive cells. As observed previously, overexpression of ERK3 strongly inhibited entry of

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**Fig. 8. Identification of a region in ERK3 conferring nuclear export activity.** A, schematic representation of the constructs. The catalytic domain of ERK3 is shaded. B, NIH 3T3 cells were transiently transfected with the indicated Myc-tagged ERK3 constructs. The localization of ectopically expressed ERK3 was determined by fluorescence microscopy after staining with anti-Myc antibody. Quantitative analysis of the localization data is presented in the bar graph, which represents the mean ± S.E. of two independent experiments. C, nuclear export assay. NIH 3T3 cells were transfected with the indicated Rev-GFP fusion constructs. After 48 h, the cells were treated for 3 h with 5 µg/ml actinomycin D to block nuclear import of Rev and 15 µg/ml cycloheximide to inhibit new protein synthesis. The cells were fixed and analyzed by fluorescence microscopy to score the percentage of cells with cytoplasmic GFP localization. Similar results were obtained in three independent experiments.

**Fig. 9. Nuclear import of ERK3 is sensitive to temperature.** NIH 3T3 cells transfected with Myc-tagged ERK3 were treated with vehicle alone or 1 ng/ml LMB for 1 h at 37 or 4°C as indicated. The localization of ERK3 was analyzed quantitatively as described in Fig. 3. The bar graph represents the mean ± S.E. of two independent experiments.

localization of a mutant ERK3 protein with alanine substitutions of lysine residues 489, 490, and 492. Mutation of these basic amino acids had no detectable influence on the cellular
cells into S phase. Notably, the ERK3-NLS (or NLS-ERK3) and NES-ERK3-NLS fusion proteins were much less effective in inducing cell cycle arrest than the wild-type kinase, suggesting that nucleocytoplasmic shuttling of ERK3 may be important for its function (Fig. 10B). To test this hypothesis further, we made an ERK3 construct in which we added both the NES and NLS to restore the shuttling of the kinase between the nuclear and cytoplasmic compartments (Fig. 10A). Overexpression of NES-ERK3-NLS was found to inhibit S phase entry to the same extent as the wild-type ERK3 protein (Fig. 10, B and C). We conclude from these results that nucleocytoplasmic shuttling of ERK3 is required for its negative regulatory action on G1 phase progression.

DISCUSSION

Nuclear Export of ERK3 by the Exportin CRM1—We report here that the atypical MAP kinase family member ERK3 constitutively localizes to the cytoplasm and nucleus in a variety of cell lines. No significant change in the subcellular distribution of ERK3 was observed in response to common mitogenic stimuli or chemical stresses. In a previous study, Cheng et al. (40) have reported that ERK3 is a constitutive nuclear kinase in
exponentially growing, quiescent, or growth factor-stimulated cells. However, these authors examined the localization of a truncated 62-kDa protein with a different C terminus, as a result of a cloning artifact, which renders the results of this study difficult to interpret. We provide compelling evidence that the cytoplasmic localization of ERK3 is dependent in large part on its active nuclear export by the CRM1 receptor. CRM1 has the broadest range of substrates among the exportin receptors and has been shown to mediate the nuclear export of numerous signaling molecules (41), including yeast and mammalian MAP kinases (18, 42–44). Our conclusion is supported by the following evidence. First, treatment of NIH 3T3 fibroblasts with LMB, a potent and selective inhibitor of CRM1 function (37, 45), causes the nuclear accumulation of ERK3 in −60% of cells. Second, overexpression of CRM1 promotes the cytoplasmic relocalization of ERK3 in a high percentage of cells. Third, overexpression of snurportin 1, which binds CRM1 with an affinity 50-fold higher than the NES-containing Rev protein (30), competes with ERK3 export, resulting in the nuclear relocalization of the kinase in around 60% of transfected cells. Finally, we show that recombinant ERK3 directly binds to CRM1 in vitro. It is noteworthy that ERK3 is the first member of the MAP kinase family for which a direct interaction with an exportin is demonstrated.

CRM1 was identified as the export receptor of proteins containing leucine-rich NESs (34–36). This export signal is defined as a short stretch of −10 amino acids with the consensus sequence LX$_{2,3}$LIVFMX$_{2}$LX(L/I) (39). However, this consensus is insufficient to describe many NESs. Indeed, a recent alignment of 58 experimentally verified NES indicated that only 36% of the sequences fit the consensus (41). In this study, we have targeted eight hydrophobic motifs in ERK3 which resemble the leucine-rich NES (Table I). Mutation of either of these sequences alone was not sufficient to change the cellular localization of ERK3. One possible explanation for these results is that nuclear export of ERK3 may be mediated by more than one NES. There are several examples of proteins containing multiple functional NES, including actin (46), Cdc25 (47), adenomatous polyposis coli (48, 49), and the STE20-like kinase MST (50). In support of this idea, we found that deletion of amino acids 399–542 leads to the nuclear accumulation of ERK3 in −25% of transfected cells, an effect that is partial compared with LMB treatment or snurportin 1 overexpression. Thus, several NESs, more or less diverging from the consensus leucine-rich NES, may interact synergistically with the CRM1 receptor and promote export. Alternatively, a novel type of export signal may mediate the binding of CRM1 to ERK3. Additional mutagenesis studies will be necessary to distinguish between these possibilities.

**Regulation of ERK3 Is Distinct from Other MAP Kinase Family Members**—Our findings highlight major differences in the regulation of ERK3 compared with other MAP kinase family members. In contrast to the classical MAP kinases ERK1/2, JNK, and p38, we observed that exposure of cells to common mitogenic stimuli or chemical stresses does not cause the relocalization of ERK3 to a distinct subcellular compartment. In the case of ERK1/2, the closest homologs of ERK3, mitogenic stimulation leads to disruption of the MEK-ERK complex (51) and promotes the rapid uptake of ERK2 into the nucleus by a facilitated transport mechanism (16). The nuclear accumulation of ERK1/2 appears to be regulated mainly by nuclear export and interaction with nuclear anchor proteins. On the opposite, exposure of cells to stress triggers the nuclear export of p38 MAP kinase to the cytoplasm in complex with phosphorylated MAP kinase-activated protein kinase-2 (43). Under the cellular conditions tested, ERK3 is constitutively localized in both the cytoplasmic and nuclear compartments.

Another regulatory feature that distinguishes ERK3 from the MAP kinases ERK1/2 is the role of activation loop phosphorylation. Previous studies have suggested that dephosphorylation of nuclear ERK1/2 in the activation loop is necessary for binding to MEK1/2 and subsequent export from the nucleus (18). Consistent with this idea, thio-phosphorylated ERK2 remains in the nucleus for much longer time than unphosphorylated ERK2 after nuclear injection (52). Interestingly, we have shown that, contrary to ERK1/2, JNK, and p38, phosphorylation of ERK3 in the activation loop (Ser-189 residue) is constitutive in various cell lines.2 This would argue against a major regulatory role of Ser-189 phosphorylation in ERK3 transport. In agreement with this, we show that replacement of Ser-189 of ERK3 by alanine or by a phosphomimetic aspartic acid does not influence the subcellular localization of the kinase in NIH 3T3 cells. Furthermore, the cellular staining pattern of Ser-189-phosphorylated ERK3 is indistinguishable from that of total ERK3.

**Nucleocyttoplasmic Shuttling of ERK3 Is Important for Regulation of Cell Cycle Progression**—The subcellular localization of MAP kinases is a critical determinant of their transcriptional and biological responses. Enforced nuclear localization of ERK1/2 is sufficient to induce morphological transformation of rodent fibroblasts (19, 20), whereas sequestration of ERK1/2 in the cytoplasm by overexpression of inactive MAP kinase phosphatase-3 inhibits Elk1-dependent transcription and progression into S phase (21). Similarly, loss of cellular adhesion inhibits nuclear accumulation of ERK1/2 and impairs Elk-1 phosphorylation (53). We have shown recently that overexpression of ERK3 inhibits S phase entry in NIH 3T3 fibroblasts (26). It was therefore of interest to determine whether the subcellular localization of the kinase may impact on its regulatory effect on the cell cycle. To this end, we engineered fusion constructs of ERK3 with the strong SV40 NLS or the MEK1 NES to force the localization of the kinase in the nucleus or cytoplasm. Importantly, we observed that enrichment of ERK3 in the nuclear or cytoplasmic compartment markedly attenuates its ability to block S phase entry. Our results strongly suggest that nucleocyttoplasmic shuttling of ERK3 is required for its negative regulatory action on cell cycle progression. Work is in progress to identify the cellular targets of ERK3.

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**REFERENCES**


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