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Regulation of Nuclear Translocation of Extracellular Signal-Regulated Kinase 5 by Active Nuclear Import and Export Mechanisms

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Extracellular signal-regulated kinase 5 (ERK5), a member of the mitogen-activated protein kinase family, plays an important role in growth factor signaling to the nucleus. However, molecular mechanisms regulating subcellular localization of ERK5 have remained unclear. Here, we show that nucleocytoplasmic shuttling of ERK5 is regulated by a bipartite nuclear localization signal-dependent nuclear import mechanism and a CRM1-dependent nuclear export mechanism. Our results show that the N-terminal half of ERK5 binds to the C-terminal half and that this binding is necessary for nuclear export of ERK5. They further show that the activating phosphorylation of ERK5 by MEK5 results in the dissociation of the binding between the N- and C-terminal halves and thus inhibits nuclear export of ERK5, causing its nuclear import. These results reveal the mechanism by which the activating phosphorylation of ERK5 induces its nuclear import and suggest a novel example of a phosphorylation-dependent control mechanism for nucleocytoplasmic shuttling of proteins.

The mitogen-activated protein kinase (MAPK) cascade, one of signaling modules ubiquitous among eukaryotes, transmits extracellular signals from cell surface receptors to specific targets within cells and regulates a wide variety of cellular functions, including cell proliferation, differentiation, and stress responses. The MAPK cascades are composed of three conserved kinases, MAPK, MAPK kinase (MAPKK), and MAPKK kinase. Extracellular stimuli, such as growth factors, induce sequential phosphorylation of the three kinases; stimulus-activated MAPKK kinase phosphorylates MAPKK, which in turn phosphorylates and activates MAPK. Phosphorylated and activated MAPK phosphorylates downstream targets, such as transcription factors, and modulates their function. To date, at least four subfamily members of the MAPK family have been identified: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun-N-terminal kinases (JNKs), p38, and ERK5. Each molecule is activated by distinct pathways and transmits signals either independently or coordinately. ERK1/2 is activated mainly by mitogenic stimuli, whereas p38 and JNK are activated mainly by stress stimuli or inflammatory cytokines (2, 6, 8, 19, 28, 31, 32, 34).

ERK5, also known as big MAP kinase 1, is activated by oxidative stress, hyperosmolarity, and several growth factors (11, 13–15, 20, 22, 23, 25, 42). Unlike other MAPK members, ERK5 has a unique large C-terminal region, whose function is not fully elucidated. MEK5 is the upstream MAPKK that specifically phosphorylates and activates ERK5 (23, 42). It has been shown that ERK5 directly interacts with, phosphorylates, and activates several transcription factors including c-Myc, Sap1a, c-Fos, Fra-1, and MEF2 family members (11, 20, 22, 35, 41). Moreover, ERK5 is shown to regulate transcription through a kinase-independent mechanism that involves its unique C-terminal half (21, 35). ERK5 is important for promoting cell proliferation (12, 23), differentiation (10), and neuronal survival (37). ERK5-null mice die around embryonic day 10, due to angiogenic failure and cardiovascular defects (30, 33, 40). Furthermore, studies with conditional ERK5 knockout mice have revealed that ERK5 plays a role in endothelial cell survival and maintenance of vascular integrity in adult mice (17). The targeted deletion of MEK5 causes early embryonic death because of cardiovascular defects (36).

As MAPK should convey extracellular signals to appropriate regions or compartments in cells, controlling subcellular localization of MAPK is vital for regulating fidelity and specificity of MAPK signaling. As many substrates of MAPK are nuclear proteins, MAPK should become localized to the nucleus to phosphorylate these nuclear proteins. For example, ERK1/2 translocates to the nucleus in response to mitogenic stimuli (7, 16, 26). Several independent mechanisms for nuclear translocation of ERK1/2 have been reported (1, 24, 27, 38). Similarly, UV irradiation and osmotic stimuli induce activation and transient nuclear localization of JNK and p38 (5, 9). Recent studies have demonstrated that expressed ERK5 localizes to the cytoplasm in quiescent cells and translocates to the nucleus when coexpressed with a constitutively active form of MEK5 (22, 39). However, the molecular mechanisms underlying the stimulus-dependent nuclear translocation of ERK5 have not been elucidated.

In this study, we have addressed the molecular mechanisms that control subcellular distribution of ERK5. We show that nuclear translocation of ERK5 is dependent on its activating phosphorylation by MEK5 and then identify a bipartite nuclear localization signal (NLS) in ERK5 that is essential for its nuclear import. Furthermore, our results show that the N-terminal half of ERK5 is bound to the C-terminal half and that this binding is required for cytoplasmic retention of ERK5. Moreover, the activating phosphorylation of the N-terminal half by MEK5 results in the disruption of the binding, causing nuclear import of ERK5. Our results further show that cytoplasmic retention of ERK5 is achieved by its nuclear export activity, which is dependent on the binding between the N- and C-

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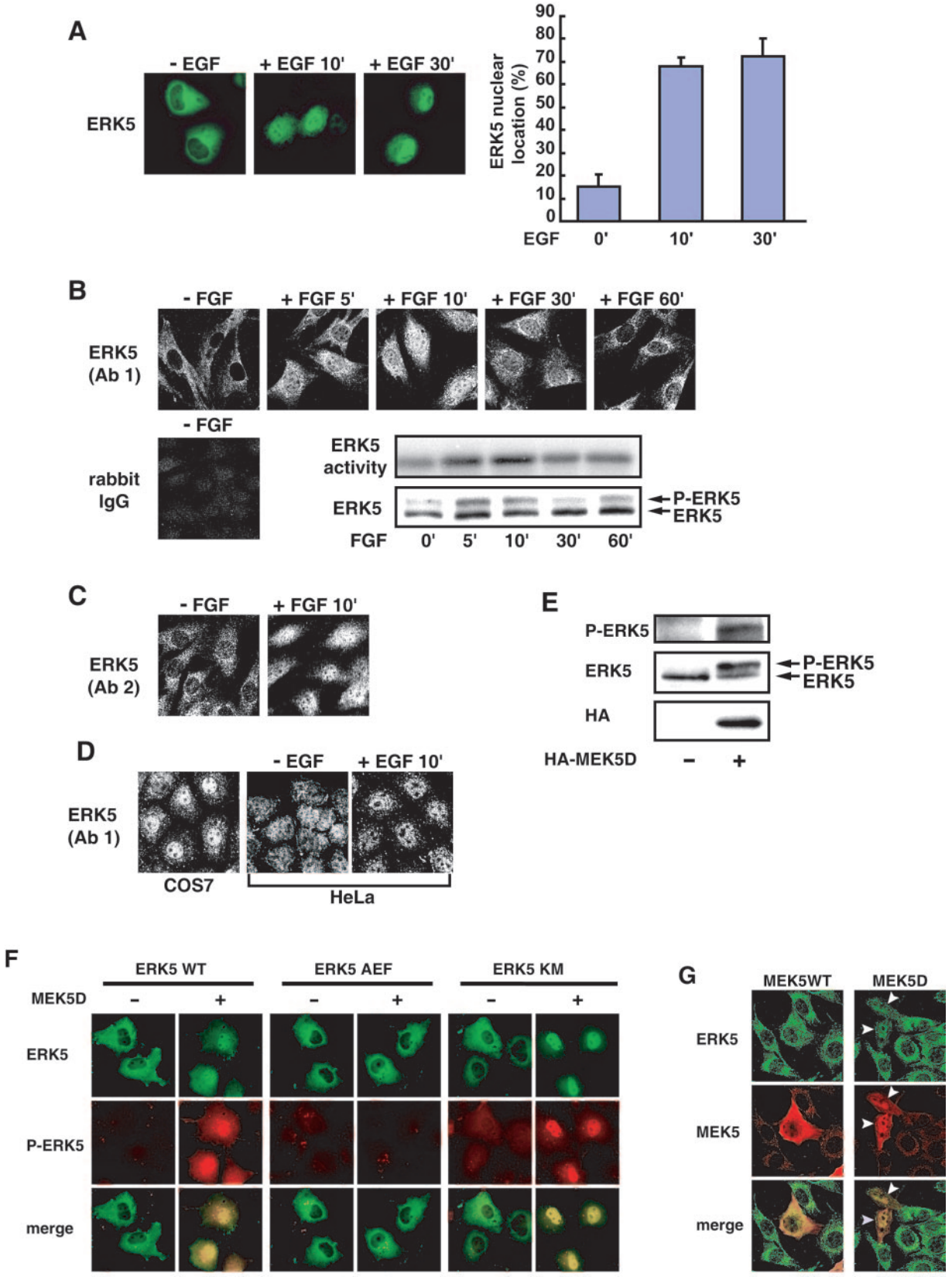

FIG. 1. Phosphorylation of ERK5 is necessary for nuclear translocation of ERK5. (A) HA-tagged ERK5 was transfected with MEK5 WT in HeLa cells. The cells were serum starved for 24 h and activated with EGF (100 ng/ml) for the indicated times. The cells were fixed and stained with anti-HA antibody. More than 130 cells were observed in terms of location of ERK5, and each percentage of cells in which staining in the

terminal halves. These results reveal a novel regulatory mechanism of subcellular localization of ERK5, which involves active nuclear import, active nuclear export, and a phosphorylation-dependent conformational change.

MATERIALS AND METHODS

Plasmids. A hemagglutinin (HA) tag was introduced into the BamHI-EcoRI sites of an expression vector (pcDNA3; Invitrogen) by ligation with the oligonucleotides 5′-GATCTGCCACCATGGCATACCCATACGACGTCCCAGATTACGCTGG ATCCG-3′ and 5′-AATTCGGATCCAGCGTAATCTGGGACGTCGTATGGG TATGCCATGGTGGCA-3′, yielding pcDNA3 HA1. Two copies of a Myc tag were introduced into the BamHI-EcoRI sites of pcDNA3 by ligation with the oligonuc leotides 5′-GATCTGCCACCATGGAGCAAAAGCTCATTTCTGAAGAGG ACTTGAATGAAATGGAGCAAAAGCTCATTTCTGAAGAGGACTTGA ATGAAGGATCCG-3′ and 5′-AATTCGGATCCTTCATTCAAGTCCTCTTCA GAAATGAGCTTTTGCTCCATTTCATTCAAGTCCTCTTCAGAAATG AGCTTTTGCTCCATGGTGGCA-3′, yielding pcDNA3 Myc1. Wild-type ERK5 (ERK5 WT), ERK5 AEF, MEK5 WT, and MEK5D have been described previously (20). The mutagenesis of ERK5 was performed using the QuikChange site-directed mutagenesis kit (Stratagene) as follows: ERK5 5 M (Lys520, Arg521, Arg522, Arg523, and Arg524 with Met), ERK5 3 M (Lys533, Arg534, and Arg535 with Met), ERK5K 8 M (Lys520, Arg521, Arg522, Arg523, Arg524, Lys533, Arg534, and Arg535 with Met), and ERK5 KM (Lys84 with Met). Several ERK5 fragments were generated by PCR. These cDNAs were subcloned into pcDNA3 HA1, pcDNA3 Myc1, and pcDNA3 Flag1 (35). pAP-1-Luc was purchased from Stratagene.

Materials and antibodies. Fibroblast growth factor (FGF) basic and epidermal growth factor (EGF) were purchased from RD Systems and BD Biosciences, respectively. Rabbit normal immunoglobulin G (IgG) and leptomycin B (LMB) were purchased from Santa Cruz and Sigma, respectively. Antibodies were purchased as follows: rabbit anti-ERK5 antibody from Sigma (Ab 1) and Calbiochem (Ab 2); rabbit anti-phospho-ERK5 antibody from Calbiochem; mouse anti-Myc (9E10), rabbit anti-Myc (A-14), and rabbit anti-glutathione *S*-transferase (anti-GST) (Z-5) antibodies from Santa Cruz; mouse anti-HA (16B12) antibody from Covance; Alexa Fluor 488–goat anti-mouse or rabbit IgG, Alexa Fluor–594 goat anti-mouse, or rabbit IgG from Molecular Probes.

Cell culture and transfection. COS7 cells, C2C12 cells, HeLa cells, and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 15% fetal bovine serum, 10% bovine calf serum, and 10% bovine calf serum, respectively. These cells were maintained in 5% CO_2 at 37°C. Cells were split onto 12-well or 60-mm dishes at 1×10^5 or 4×10^5 cells per dish, respectively. The COS7 cells, C2C12 cells, and NIH 3T3 cells were transfected by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. The HeLa cells were transfected by using FuGENE 6 reagent (Roche) according to the manufacturer's protocol.

Immunostaining. After 24 h of transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. After being blocked with 3% bovine serum albumin in PBS, the cells were incubated with indicated primary antibodies for 16 h at 4°C and then incubated with the appropriate secondary antibodies for 1 h at room temperature. Cells were finally mounted in Mowiol and examined with a laser scanning confocal microscope (Bio-Rad) or Axiophoto2 (Zeiss).

Luciferase assay. Cells split onto 12-well dishes were harvested for assay 24 h after transfection. The luciferase activity in cell lysates was measured by the luciferase assay system (Promega). To determine transfection efficiency, coexpressed beta-galactosidase activity was measured, and the data were normalized for beta-galactosidase activities. The data represent means and standard deviations of at least three independent experiments.

Immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer (50 mM HEPES [pH 7.3], 2 mM EGTA, 2 mM EDTA, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 17-μg/ml aprotinin, 1 mM vanadate, 1% protein inhibitor cocktail [Sigma], 1% NP-40, and 10% glycerol). Tagged proteins were immunoprecipitated from cell lysates by incubation with indicated primary antibodies and protein G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. The precipitates were washed twice with lysis buffer and then eluted by incubation with elution buffer (150 mM glycine Cl, pH 2.2). In some cases, cells were lysed in 1× Laemmli sample buffer (62.5 mM Tris-Cl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromophenol blue). The proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting.

Kinase assay. Cells were lysed in lysis buffer. Endogenous ERK5 or tagged-ERK5 was immunoprecipitated with anti-ERK5 antibody (Ab 1) or anti-tag antibody and incubated for 10 min at 30°C with myelin basic protein (final concentration, 2 mg/ml) in reaction buffer (20 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, and 100 μM ATP, or 2 μCi of [γ-^{32}P]ATP).

Preparation of proteins and GST pull-down assay. GST-ERK5N, which was constructed by using pGEX6P1 (Amersham Pharmacia Biotech), was expressed in *Escherichia coli* and purified by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). His-ERK5C was constructed by using the pFastBac HT-B vector and the Bac-to-Bac system (Invitrogen), expressed in Sf9 cells and purified by ProBond Resin (Invitrogen). The GST-ERK5N protein and the His-ERK5C protein were mixed and then incubated with glutathione-Sepharose 4B in a binding buffer (50 mM HEPES [pH 7.3], 2 mM EGTA, 2 mM EDTA, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 17-μg/ml aprotinin, 1 mM vanadate, 1% protein inhibitor cocktail [Sigma], and 10% glycerol) for 4 h at 4°C. The precipitates were then washed twice with the binding buffer. The proteins were separated by SDS-PAGE and analyzed by immunoblotting.

Preparation of RNA samples and RT-PCR analysis. Whole-cell RNA was extracted by using an RNeasy mini kit according to the manufacturer's instructions (QIAGEN). Total RNA was then reverse transcribed into cDNA by using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligonucleotide random hexamers. Prepared cDNA was purified and subjected to quantitative PCR analysis with the Light Cycler (Roche Diagnostics) with a SYBR Green PCR kit (QIAGEN). The primers for the PCR analysis were as follows: for c-Fos, 5′-GTAGAGCAGCTATCTCCTGA-3′ and 5′-CTTCTCATCTTC AAGTTGATC-3′; for glyceraldehyde-3-phosphate dehydrogenase, 5′-CATCCA CTGGTGCTGCCAAGGCTGT-3′ and 5′-ACAACCTGGTCCTCAGTGTAG CCCA-3′. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA.

RESULTS

Phosphorylation of ERK5 is necessary for its nuclear translocation. Nuclear translocation of expressed ERK5 has been reported to occur when coexpressed with constitutively active MEK5. Thus, we first examined whether expressed ERK5 translocates to the nucleus upon stimulation by natural ligands.

nucleus was stronger than that in the cytoplasm is shown (right). Representative images of the cells are shown (left). (B) C2C12 cells were serum starved for 24 h and activated with FGF (50 ng/ml) for the indicated times. The cells were fixed, stained with anti-ERK5 antibody (Ab 1; Sigma) or with normal rabbit IgG, and examined with a confocal laser microscope (top). The cells were harvested with lysis buffer and subjected to the immunoblotting with anti-ERK5 antibody or subjected to the kinase assay to measure ERK5 activity (bottom). (C) C2C12 cells were serum starved for 24 h and activated with FGF (50 ng/ml) for the indicated times. The cells were fixed, stained with anti-ERK5 antibody (Ab 2; Calbiochem), and examined with a confocal laser microscope. (D) COS7 and HeLa cells were serum starved for 24 h. HeLa cells were treated with EGF (100 ng/ml). The cells were then fixed, stained with anti-ERK5 antibody (Ab 1; Sigma), and examined with a confocal laser microscope. (E) The HA-tagged MEK5D was transfected in COS7 cells. After 24 h, the cells were serum starved for 24 h. The cells were harvested with Laemmli sample buffer and subjected to the immunoblotting with anti-ERK5, anti-phospho-ERK5, and anti-HA antibodies. (F) The indicated HA-tagged ERK5 constructs were transfected with or without MEK5D in COS7 cells. After 24 h, the cells were fixed and stained with anti-HA antibody (green) and anti-phospho-ERK5 antibody (red). (G) The indicated Myc-tagged MEK5 constructs were transfected in C2C12 cells. After 24 h, the cells were fixed, stained with anti-Myc antibody (red) and anti-ERK5 antibody (Ab 1) (green), and examined with a confocal laser microscope.

We coexpressed ERK5 WT and MEK5 WT in HeLa cells and stimulated the cells by treatment with EGF. The EGF treatment caused nuclear translocation of ERK5 (Fig. 1A, left). Semiquantitation demonstrated that the percentage of the cells which showed strong nuclear accumulation of ERK5 increased to 68% and 72% after 10 min and 30 min of stimulation, respectively, from 15% before stimulation (Fig. 1A, right). These results clearly show that expressed ERK5 was able to translocate to the nucleus upon stimulation by natural ligands. There have been apparently contradictory reports concerning the subcellular localization of endogenous ERK5. One report showed that endogenous inactive ERK5 localizes in the cytoplasm in MCF7 and NP9 cells and translocates to the nucleus upon stimulation in MCF7 cells (14). However, the same report also showed that ERK5 localizes mainly in the nucleus without stimulation in SKBR3 cells (14). Moreover, another report showed that ERK5 localizes in the nucleus before stimulation in several cell lines, including COS7 and HeLa cells (29). Then, we next examined subcellular distribution of endogenous ERK5 in several cell lines by immunostaining. Endogenous ERK5 localized in the cytoplasm after serum starvation and translocated to the nucleus in response to FGF stimulation in C2C12 cells (Fig. 1B, top). ERK5 began to accumulate in the nucleus within 5 min after FGF treatment and then began to relocalize to the cytoplasm within 30 min. This nuclear accumulation of ERK5 paralleled the phosphorylation and kinase activity of ERK5 (Fig. 1B, bottom). The use of normal rabbit IgG instead of anti-ERK5 antibody almost completely abolished staining (Fig. 1B), and staining with another anti-ERK5 antibody (against a different epitope of ERK5) gave essentially the same results (Fig. 1C), suggesting the accuracy of the immunostaining against endogenous ERK5. A similar behavior of ERK5 was also observed in MCF7 cells after neuregulin stimulation (data not shown). On the other hand, endogenous ERK5 localized mainly in the nucleus even before stimulation in COS7 and HeLa cells (Fig. 1D), as previously reported (29). Interestingly, nuclear accumulation of endogenous ERK5 in HeLa cells was enhanced in response to stimulation with EGF (Fig. 1D). We could not detect marked changes in the subcellular localization of endogenous ERK5 in COS7 cells by treatment with EGF or FGF (data not shown). Thus, subcellular localization of endogenous ERK5 depends on the cell type. Our results, however, indicate that endogenous ERK5, like expressed ERK5, is able to translocate to the nucleus upon stimulation, suggesting that there should be a molecular mechanism regulating the stimulus-dependent nuclear translocation of ERK5. Thus, we examined whether MEK5 phosphorylation of ERK5 was necessary for its nuclear translocation. We used two mutants of ERK5, ERK5 AEF and ERK5 KM. ERK5 AEF is an nonphosphorylatable mutant, in which threonine and tyrosine residues at the activating phosphorylation sites (TEY) are replaced by alanine and phenylalanine, respectively. ERK5 KM is a kinase-deficient mutant, in which lysine 84 is replaced by methionine. We utilized MEK5D, a constitutively active form of MEK5 (20), to induce activating phosphorylation of expressed ERK5. Expression of MEK5D resulted in strong phosphorylation of endogenous ERK5 in COS7 cells (Fig. 1E). We expressed ERK5 WT, ERK5 AEF, or ERK5 KM in COS7 cells with or without MEK5D and examined their subcellular localization by immunostaining. Every form of ERK5 localized in the cytoplasm without MEK5D. When coexpressed with MEK5D, ERK5 WT and ERK5 KM, but not ERK5 AEF, translocated to the nucleus (Fig. 1F). Moreover, immunostaining against the phosphorylated form of ERK5 revealed that both ERK5 WT and ERK5 KM were phosphorylated on their TEY sequence (Fig. 1F). These results indicate that the activating phosphorylation of ERK5 on the TEY sequence by MEK5, but not the kinase activity of ERK5, is necessary for ERK5 nuclear translocation. To examine whether expression of MEK5D induces nuclear translocation of endogenous ERK5, we expressed MEK5 WT and MEK5D in C2C12 cells and examined the subcellular localization of endogenous ERK5 by immunostaining. While expression of MEK5 WT did not affect the cytoplasmic localization of ERK5 (Fig. 1G), expression of MEK5D induced nuclear translocation of endogenous ERK5 (Fig. 1G, arrowheads). Thus, these results suggest that the activating phosphorylation is necessary for nuclear translocation of endogenous ERK5, as well as expressed ERK5.

Identification of a bipartite nuclear localization signal in ERK5. It has previously been shown that a portion of mouse ERK5, residues 505 to 539, is necessary for its nuclear translocation (39). As this region contains two clusters of basic amino acids in tandem, residues 520 to 524 (KRRRR) and residues 533 to 535 (KRR), we hypothesized that this region functions as a bipartite NLS sequence. We generated several mutant forms of ERK5, in which the basic amino acids in the first cluster, second cluster, or both clusters were mutated to methionines (Fig. 2A), and we then expressed these mutants or ERK5 WT with or without MEK5D. ERK5 WT and each mutant form of ERK5 localized in the cytoplasm when expressed without MEK5D. While coexpression of MEK5D resulted in the nuclear translocation of ERK5 WT, all the mutant forms of ERK5 remained in the cytoplasm (Fig. 2B). This result indicates that both of the basic amino acid clusters are required for ERK5 to enter the nucleus and thus demonstrates that this region functions as a bipartite NLS sequence. We have previously shown that activation of ERK5 induces activation of the activator protein 1 (AP-1) transcriptional complex, one of the downstream targets of ERK5 (35). We then examined whether these nuclear translocation-deficient mutants of ERK5 are able to stimulate the AP-1 activity by using a reporter assay measuring the transcriptional activity of AP-1. While expression of ERK5 WT with MEK5D increased AP-1 activity, expression of each mutant form of ERK5 with MEK5D did not (Fig. 2C). There was no significant difference in the expression levels of MEK5D and each mutant form of ERK5 (Fig. 2C, inset). Moreover, there were no significant differences in the phosphorylation level and kinase activity between ERK5 WT and the NLS mutants (Fig. 2C, inset). These results indicate that translocation of ERK5 to the nucleus is necessary for activation of the AP-1 activity by the MEK5-ERK5 pathway. The MEK5-ERK5 pathway has been shown to induce expression of c-Fos (20). To further analyze the physiological relevance of nuclear translocation of ERK5, we examined whether the NLS mutant of ERK5 is able to induce expression of endogenous c-*fos* mRNA. While expression of ERK5 WT with MEK5D induced c-Fos mRNA expression, expression of ERK5 8 M with MEK5D did not (Fig. 2D).

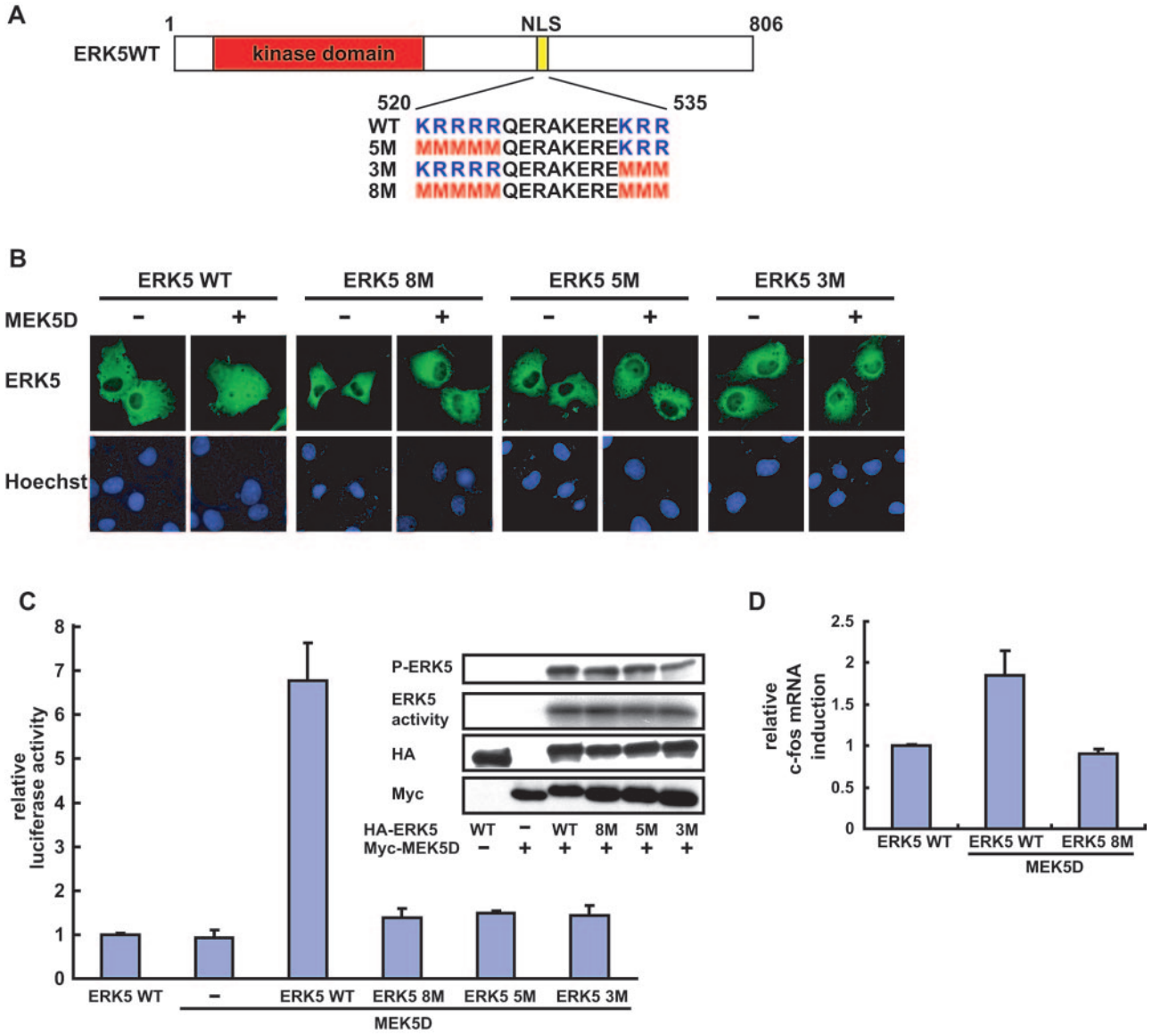

FIG. 2. Identification of a bipartite nuclear localization signal in ERK5. (A) Schematic representation of mouse ERK5 and several mutant forms of ERK5 in which the basic amino acids in the first cluster, second cluster, or both clusters were mutated to methionines. (B) The indicated HA-tagged ERK5 constructs were transfected with or without MEK5D in COS7 cells. After 24 h, the cells were fixed and stained with anti-HA antibody (green) and Hoechst 33342 (blue) for nuclear staining. (C) The indicated expression plasmids were transfected with pAP1-Luc, the reporter plasmid, in COS7 cells. After 24 h, the cells were harvested, and then lysates were subjected to a luciferase assay. The cells were harvested with lysis buffer and subjected to the immunoblotting with anti-HA, anti-Myc, and anti-phospho-ERK5 antibodies or subjected to the kinase assay to measure ERK5 activity (inset). (D) The indicated expression plasmids were transfected in NIH 3T3 cells. After 24 h, the cells were collected, and the relative levels of endogenous c-*fos* mRNA were determined by RT-PCR analysis. As transfection efficiency was about 20%, the increase of c-*fos* mRNA of about 1.8 fold by ERK5 WT-MEK5D implies that the induction was about 5 fold in the transfected cells.

Taken together, these results suggest that nuclear translocation is important for ERK5 function.

Interaction of the N-terminal-half domain of ERK5 with the C-terminal-half domain. In unstimulated conditions, ERK5 is in the cytoplasm, although it has the NLS, which is required for the phosphorylation-dependent nuclear import of ERK5. To find a clue to a mechanism regulating cytoplasmic localization of unphosphorylated ERK5, we made two deletion constructs of ERK5, ERK5N (residues 1 to 407) and ERK5C (residues 401 to 806). ERK5N, an N-terminal half of ERK5, has a kinase domain, and ERK5C, a C-terminal half of ERK5, has the bipartite NLS (Fig. 3A). When expressed in COS7 cells, ERK5N was distributed in both the nucleus and the cytoplasm, whereas ERK5C localized in the nucleus (Fig. 3B). The pancellular localization of ERK5N suggests that ERK5N was diffusively distributed without active transport, and the nuclear localization of ERK5C indicated that the bipartite NLS is functional. We then examined the effect of coexpression of ERK5N and ERK5C on their localization. Remarkably, coexpression of ERK5N and ERK5C caused a dramatic change in

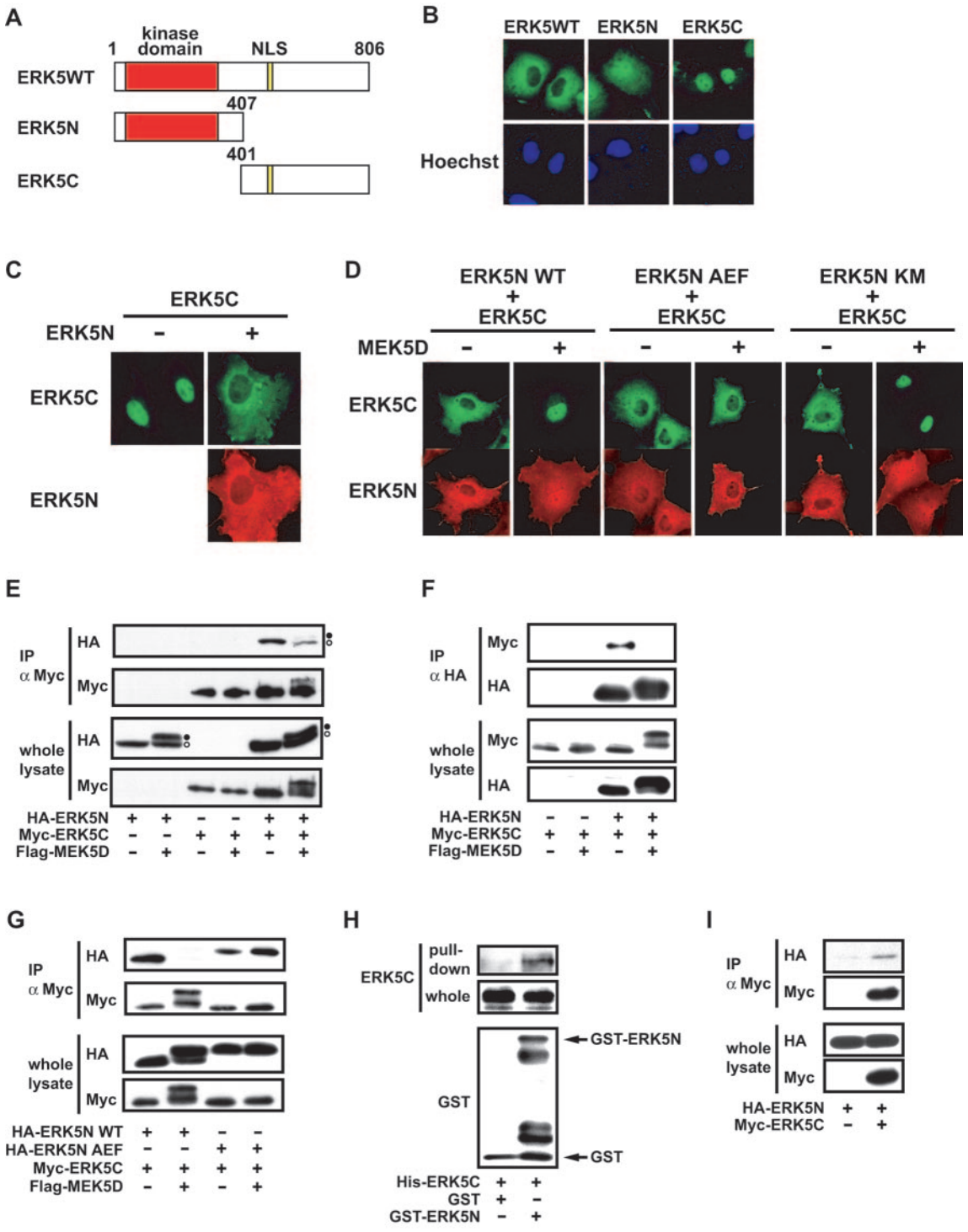

FIG. 3. The N-terminal half of ERK5 binds to its C-terminal half. (A) Mouse ERK5 and ERK5 deletion mutants used in these experiments. (B) COS7 cells were transfected with the indicated HA-tagged ERK5 constructs. After 24 h, the cells were fixed and stained with anti-HA antibody (green) and Hoechst 33342 (blue). (C) HA-tagged ERK5C was transiently coexpressed with or without Myc-tagged ERK5N in COS7 cells. After 24 h, the cells were fixed and stained with anti-HA antibody (green) and anti-Myc antibody (red). (D) HA-tagged ERK5C and the indicated Myc-tagged ERK5N constructs were coexpressed with or without MEK5D in COS7 cells. After 24 h, the cells were fixed and stained with anti-HA antibody (green) and anti-Myc antibody (red). (E to G) COS7 cells were transiently cotransfected with the indicated expression plasmids. Lysates from these cells were subjected to SDS-PAGE (whole) or immunoprecipitation with anti-Myc antibody or anti-HA antibody (IP). Coimmunoprecipitated proteins were detected by immunoblotting with anti-HA and anti-Myc antibodies. (H) The ERK5C protein was mixed with GST protein or the GST fused ERK5N protein. The mixture was subjected to SDS-PAGE (whole) or GST pull-down assay (pull-down). Coimmunoprecipitated ERK5C was detected by immunoblotting with anti-ERK5 antibody. The amount of GST or GST-ERK5N added in the mixture was detected by immunoblotting with anti-GST antibody (GST or GST-ERK5N). (I) The lysates of COS7 cells transfected with HA-ERK5N were

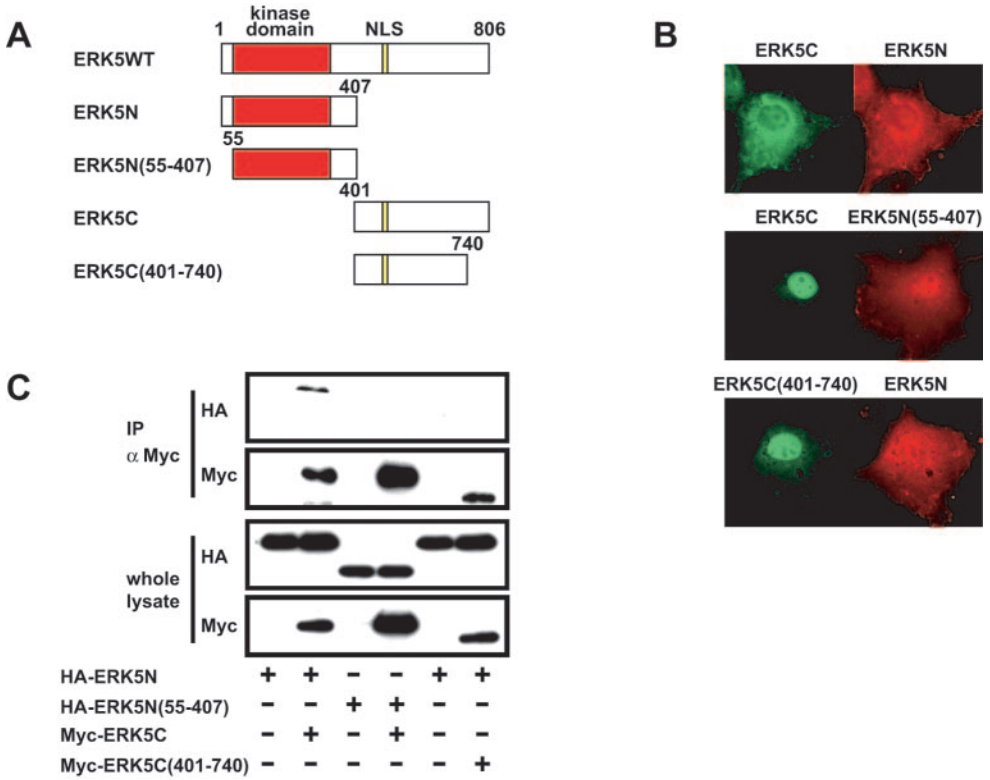

FIG. 4. Identification of regions required for the binding in ERK5. (A) Schematic representation of ERK5 constructs used in these experiments. (B) COS7 cells were cotransfected with HA-tagged ERK5C constructs and Myc-tagged ERK5N constructs, and subcellular distribution of these constructs was examined by immunostaining with anti-HA antibody (green) and anti-Myc antibody (red). (C) COS7 cells were transiently transfected with the indicated expression plasmids. Lysates from these cells were subjected to SDS-PAGE (whole) or immunoprecipitation with anti-Myc antibody (IP). Following SDS-PAGE, the samples were immunoblotted with anti-HA and anti-Myc antibodies.

their distribution; both ERK5N and ERK5C were excluded from the nucleus (Fig. 3C). ERK5N and ERK5C apparently colocalized in the cytoplasm, suggesting that ERK5N binds to ERK5C (see below). The cytoplasmic localization of this putative ERK5N/ERK5C complex is in agreement with the cytoplasmic localization of unphosphorylated full-length ERK5 WT. This suggests that the N-terminal half of full-length ERK5 interacts intramolecularly with its C-terminal half. Interestingly, expression of MEK5D in cells expressing ERK5N and ERK5C resulted in nuclear accumulation of ERK5C and both nuclear and cytoplasmic localization of ERK5N (Fig. 3D), suggesting that the activating phosphorylation of ERK5N by MEK5D disrupts the colocalization, probably the association, of ERK5C with ERK5N. Then, we coexpressed ERK5N AEF or ERK5N KM, instead of ERK5N WT, together with ERK5C. In both cases, ERK5C localized in the cytoplasm (Fig. 3D). Expression of MEK5D resulted in the relocation of ERK5C to the nucleus in the case of ERK5N KM expression, but not in the case of ERK5N AEF expression (Fig. 3D). Again, this situation was the same as that for full-length ERK5 (Fig. 1F). Thus, these results suggest that ERK5N and ERK5C form a complex whose dissociation is induced by the activating phosphorylation of ERK5N by MEK5. To test this possibility, we performed immunoprecipitation experiments. HA-tagged ERK5N was coexpressed with Myc-tagged ERK5C in COS7 cells, and the cell lysates were subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting with anti-HA antibody. ERK5N was coprecipitated with ERK5C (Fig. 3E), suggesting that ERK5N and ERK5C form a complex. When coexpressed with MEK5D, more than half of ERK5N underwent phosphorylation, showing a mobility-shifted band (Fig. 3E, HA, closed circle). This phosphorylated ERK5N was not coprecipitated with ERK5C (Fig. 3E). Only a small amount of unphosphorylated ERK5N (open circle) was coprecipitated with ERK5C in this case (Fig. 3E, IP, HA). These results further support our idea that MEK5-induced phosphorylation of ERK5N leads to the dissociation of the ERK5N/ERK5C complex. The mobility-shifted band of ERK5C in Fig. 3E may be caused by phosphorylation of ERK5C by ERK5N (our unpublished data). Similar results were obtained when the cell lysates were subjected to immunoprecipitation with anti-HA antibody (Fig. 3F). To further examine the role of phos-

mixed with the lysates of COS7 cells transfected with Myc-ERK5N or an empty vector. The mixture was subjected to SDS-PAGE (whole) or immunoprecipitation with anti-Myc antibody (IP). Coimmunoprecipitated proteins were detected by immunoblotting with anti-HA and anti-Myc antibodies.

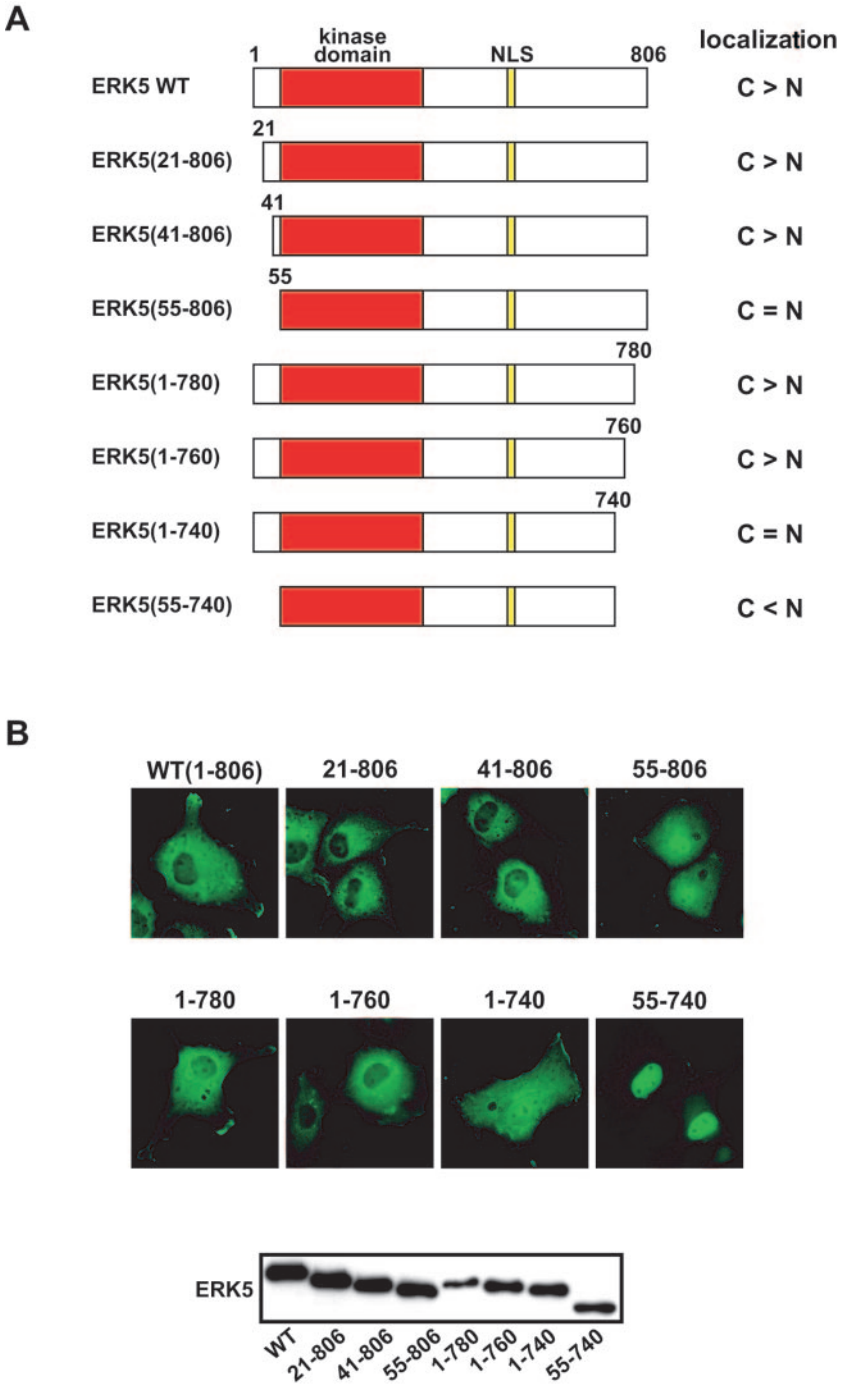

FIG. 5. Two regions, residues 41 to 54 and residues 741 to 760, are important for cytoplasmic localization of ERK5. (A) (Left) Schematic representations of ERK5 constructs used in these experiments. (Right) Summary of subcellular localization of ERK5 constructs shown in panel B, labeled as uniform distribution between the cytoplasm and the nucleus (C = N), predominantly nuclear distribution (C < N), or predominantly cytoplasmic distribution (C > N). (B) COS7 cells were transiently expressed with HA-tagged ERK5 constructs. After 24 h, the cells were fixed and stained with anti-HA antibody (top). The cells were harvested with Laemmli sample buffer and subjected to immunoblotting with anti-HA antibody (bottom).

phorylation of ERK5N in promoting a conformational change of ERK5, we performed immunoprecipitation experiments using the ERK5N AEF mutant instead of ERK5N WT. Unlike ERK5N WT, ERK5N AEF was coimmunoprecipitated with ERK5C even in the presence of MEK5D (Fig. 3G), suggesting that the activating phosphorylation of ERK5N by MEK5D is necessary for the dissociation of the ERK5N/ERK5C complex. These results are thus consistent with the suggestions that the N-terminal half of ERK5 interacts with its C-terminal half, that this interaction is required for the cyto-

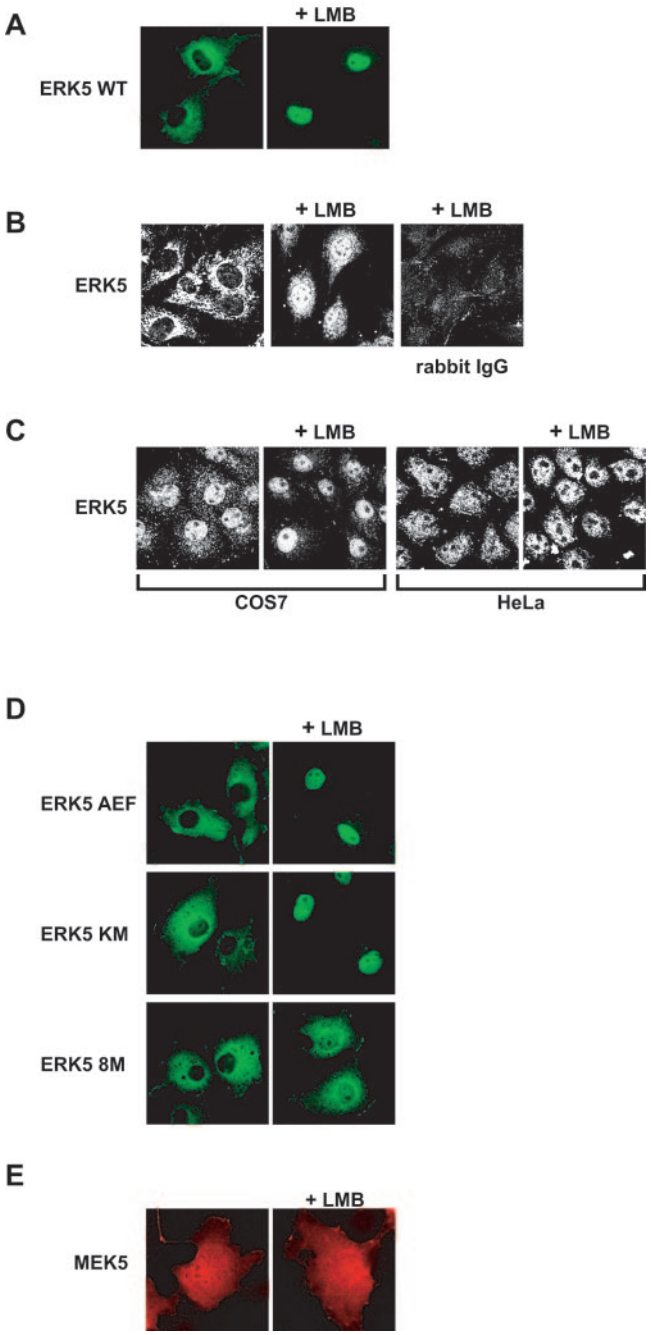

FIG. 6. Subcellular localization of ERK5 is regulated by a CRM1-dependent nuclear export mechanism. (A) COS7 cells were expressed with HA-tagged ERK5. After 24 h, the cells were treated with or without LMB (5 ng/ml) for 1 h, fixed, and stained with anti-HA antibody. (B) C2C12 cells were treated with LMB as in panel A. The cells were fixed and stained with anti-ERK5 antibody (Ab 1) and examined with a confocal laser microscopy. (C) COS7 cells and HeLa cells were treated with LMB as in panel A. The cells were fixed and stained with anti-ERK5 antibody (Ab 1) and examined with a confocal laser microscopy. (D) COS7 cells expressing ERK5 AEF, ERK5 KM or ERK5 8 M mutants were treated as in panel A. (E) HA-tagged MEK5 was transiently expressed in COS7 cells. After 24 h, the cells were treated with LMB as in panel A, fixed, and stained with anti-HA antibody.

plasmic localization of ERK5, and that the phosphorylation of the TEY sequence in the N-terminal kinase domain by MEK5 causes disruption of the interaction, resulting in nuclear import of ERK5. To confirm that the N-terminal half of ERK5 interacts directly with the C-terminal half, we prepared the GST-fused ERK5N protein and the His-tagged ERK5C protein and examined their interaction by GST pull-down assay. The ERK5C protein was efficiently coprecipitated with the GST-ERK5N protein (Fig. 3H), indicating that the two halves interact directly. Moreover, when immunoprecipitation was performed with each half of ERK5, which was separately expressed in COS7 cells, ERK5N was coimmunoprecipitated with ERK5C (Fig. 3I). This result further confirms the direct interaction of ERK5N with ERK5C.

To identify a region in ERK5N and ERK5C responsible for the interaction, we generated two additional constructs of ERK5, ERK5N (residues 55 to 407) and ERK5C (residues 401 to 740) (Fig. 4A). When ERK5C was coexpressed with ERK5N (residues 55 to 407), they did not colocalize, and ERK5C remained in the nucleus (Fig. 4B). Similarly, coexpression of ERK5N with ERK5C (residues 401 to 740) did not induce their colocalization in the cytoplasm. The immunoprecipitation assay showed that ERK5C was not coprecipitated with ERK5N (residues 55 to 401), and ERK5C (residues 401 to 740) was not coprecipitated with ERK5N (Fig. 4C). These results indicate that an N-terminal region (residues 1 to 54) and a C-terminal region (residues 741 to 806) are necessary for the interaction. Next, we further generated several N-terminal- and/or C-terminal-deleted constructs of full-length ERK5 (Fig. 5A), and examined their subcellular localization. Like full-length ERK5, ERK5 (residues 21 to 806), ERK5 (residues 41 to 806), ERK5 (residues 1 to 780), and ERK5 (residues 1 to 760) localized in the cytoplasm (Fig. 5B, top). In contrast, both ERK5 (residues 55 to 806) and ERK5 (residues 1 to 740) localized in the nucleus, as well as in the cytoplasm. Notably, ERK5 (residues 55 to 740) predominantly localized in the nucleus. The expression levels of these constructs were similar (Fig. 5B, bottom). These results suggest that two regions, residues 41 to 54 and residues 741 to 760, are important for cytoplasmic localization, and probably the intramolecular interaction, of ERK5. It should be noted that our immunoprecipitation experiment did not detect dimer or oligomer formation of full-length ERK5 (data not shown), suggesting intramolecular interaction, not intermolecular interaction, in ERK5.

Subcellular localization of ERK5 is regulated by a CRM1-dependent nuclear export mechanism. Recent reports showed that treatment with LMB, a specific inhibitor of the nuclear export receptor CRM1, induces nuclear accumulation of expressed ERK5 (3, 29). We confirmed this observation (Fig. 6A). Furthermore, LMB treatment resulted in translocation of endogenous ERK5 from the cytoplasm to the nucleus in C2C12 cells, even under unstimulated conditions (Fig. 6B). LMB treatment enhanced the nuclear accumulation of endogenous ERK5 even in COS7 cells and HeLa cells (Fig. 6C), indicating that a portion of endogenous ERK5 in these cells also localizes in the cytoplasm by a CRM1-dependent nuclear export activity. These results suggest that subcellular localization of endogenous ERK5 in many cell lines is regulated by a CRM1-dependent active nuclear export mechanism. Then, we examined the effect of LMB treatment on subcellular distribu-

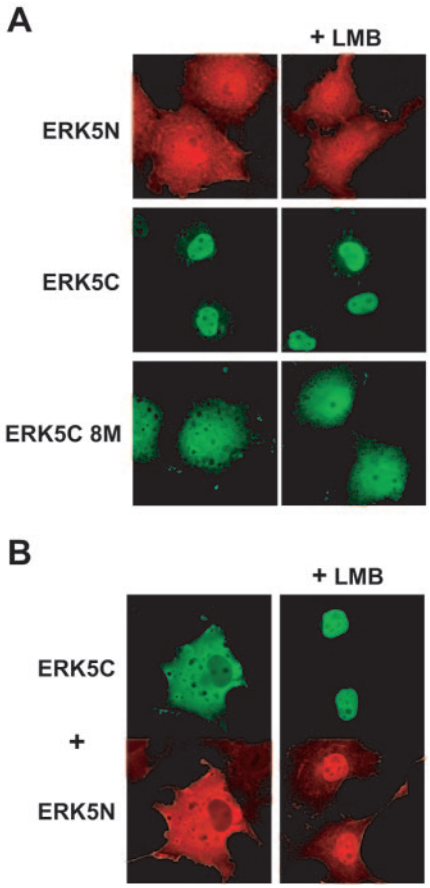

FIG. 7. Both the N- and C- terminal halves of ERK5 are required for the nuclear export activity of ERK5. (A) COS7 cells were expressed with Myc-tagged ERK5N, HA-tagged ERK5C, or HA-tagged ERK5 8 M. After 24 h, the cells were treated with or without LMB (5 ng/ml) for 1 h, fixed, and stained with anti-HA (green) or anti-Myc (red) antibody. (B) COS7 cells were coexpressed with HA-tagged ERK5C and Myc-tagged ERK5N. After 24 h, the cells were treated as shown in panel A.

tion of several mutants of ERK5. Both ERK5 AEF and ERK5 KM translocated to the nucleus upon LMB treatment (Fig. 6D), suggesting that the LMB treatment-dependent nuclear accumulation of ERK5 does not require the activating phosphorylation of ERK5 or the kinase activity of ERK5. In fact, LMB treatment did not cause the phosphorylation of ERK5 (data not shown). In contrast, ERK5 8M, the NLS-deficient mutant, failed to translocate to the nucleus upon LMB treatment (Fig. 6D), indicating that the LMB-induced nuclear localization of ERK5 is dependent on its NLS. These results taken together suggest that the bipartite NLS of ERK5 is functionally active even in unphosphorylated, full-length ERK5 and that the nuclear export activity of ERK5 is stronger than the NLS activity under unstimulated conditions, leading to the cytoplasmic localization of ERK5. Moreover, these results further suggest that the effect of activating phosphorylation of ERK5 by MEK5 is to inhibit the nuclear export of ERK5 rather than to promote its nuclear import. Thus, it is possible that the role of intramolecular interaction in unphosphorylated ERK5 is to expose or form a region responsible for nuclear export and that the phosphorylation-induced disruption of the interaction would cause masking or disruption of the region, resulting in the nuclear translocation of ERK5. The subcellular localization of MEK5 was not affected by LMB treatment (Fig. 6E).

To find out a region in ERK5 responsible for its nuclear export, we examined the effect of LMB treatment on subcellular distribution of ERK5N and ERK5C. Surprisingly, neither ERK5N nor ERK5C changed its subcellular localization upon LMB treatment (Fig. 7A). As ERK5C strongly accumulated in the nucleus due to its NLS even before LMB treatment, it might be difficult to see the effect of LMB on localization of ERK5C. Then, we made ERK5C 8M, in which the NLS was completely disrupted. ERK5C 8M localized in both the cytoplasm and the nucleus, and LMB treatment did not cause nuclear accumulation of ERK5 8M (Fig. 7A). These results demonstrate that neither the N-terminal-half domain of ERK5 alone nor the C-terminal-half domain alone contains nuclear export signal (NES) activity. We then coexpressed ERK5N with ERK5C. Interestingly, both ERK5N and ERK5C translocated to the nucleus from the cytoplasm upon LMB treatment (Fig. 7B), like full-length ERK5 (Fig. 6). The binding between ERK5N and ERK5C was not affected by LMB treatment (data not shown). Taken together, these results indicate that both the N-terminal half of ERK5 and the C-terminal half are required for the nuclear export of ERK5 and suggest that the intramolecular interaction of the N-terminal half with the C-terminal half in ERK5 produces an NES.

DISCUSSION

In this study, we examined the molecular mechanisms regulating the subcellular localization of ERK5. We first show that endogenous ERK5, as well as expressed ERK5, translocates to the nucleus from the cytoplasm by a stimulus-dependent mechanism. Our results then show that subcellular localization of ERK5 is regulated by both active nuclear import and export mechanisms. They further show that the direct interaction between the N-terminal half of ERK5 and its C-terminal half is important for cytoplasmic localization of ERK5 and that the activating phosphorylation of the N-terminal half of ERK5 by MEK5 disrupts the interaction, resulting in nuclear accumulation of ERK5. Moreover, deletion of the regions responsible for the interaction causes nuclear translocation of ERK5 without stimulation. Furthermore, the results in this study demonstrate that cytoplasmic localization of ERK5 is regulated by its nuclear export activity and suggest that intramolecular interaction in ERK5 is required for its nuclear export activity. Based on these results, we propose a hypothetical model for the molecular mechanism regulating subcellular localization of ERK5 (Fig. 8). In unstimulated conditions, the N-terminal half of ERK5 interacts intramolecularly with its C-terminal half, forming a region responsible for the nuclear export activity that is sensitive to LMB. Although the NLS in ERK5 is active in this state, nuclear export activity is stronger than NLS activity, resulting in cytoplasmic localization of ERK5 (Fig. 8A or B; see below). Upon stimulation, the activating phosphorylation at the TEY sequence in the N-terminal half of ERK5 by MEK5 disrupts the intramolecular interaction in ERK5 and thus inhibits the nuclear export activity of ERK5. Therefore,

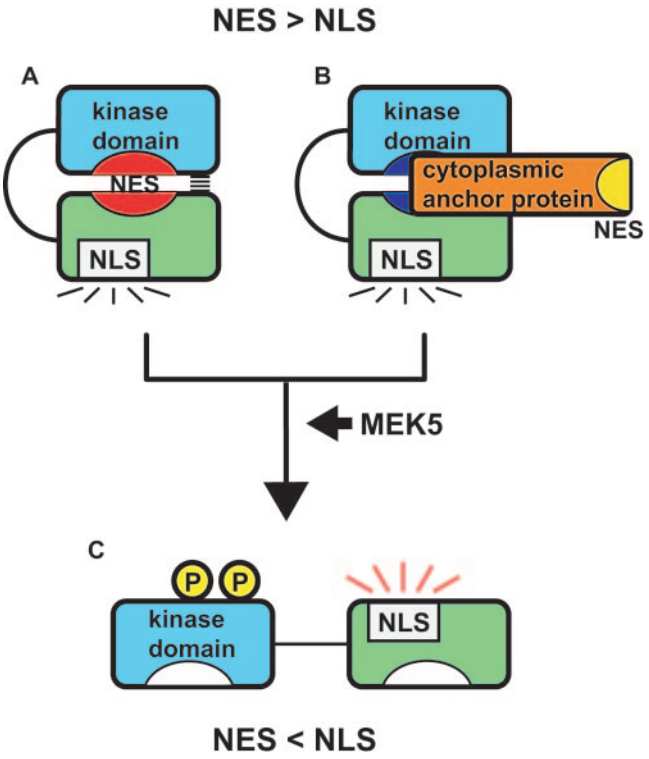

FIG. 8. A hypothetical model for the regulatory mechanism of subcellular localization of ERK5. In the resting state, the N-terminal half of ERK5 interacts intramolecularly with its C-terminal half, forming an NES (A) or a region responsible for the interaction with a cytoplasmic anchor protein that contains an NES (B). Although the NLS in ERK5 is active in this state, NES activity is stronger than NLS activity, resulting in cytoplasmic localization of ERK5. Upon stimulation, the activating phosphorylation at the TEY sequence in the N-terminal half of ERK5 by MEK5 disrupts intramolecular interaction in ERK5 and thus inhibits the nuclear export activity of ERK5. Therefore, ERK5 translocates to the nucleus because the NLS is constitutively active (C). The circled P represents phosphorylation.

ERK5 translocates to the nucleus because the NLS is constitutively active (Fig. 8C).

Our results show that cytoplasmic localization of ERK5 is regulated by active nuclear export mechanisms. There may be two possibilities. One is that ERK5 itself has an NES (Fig. 8A), and the other is that ERK5 binds to a cytoplasmic anchor protein having NES (Fig. 8B). Our results show that neither the N-terminal nor the C-terminal half of ERK5 alone has nuclear export activity. Only when the N-terminal half of ERK5 is coexpressed with the C-terminal half did the complex of the N- and C- terminal half of ERK5 have nuclear export activity. In addition, deletion mutants of ERK5 which lack intramolecularly interacting regions failed to localize in the cytoplasm, implying that these mutants lack their nuclear export activity. It is possible, therefore, that a putative NES or a region responsible for binding to a cytoplasmic anchor protein in ERK5 is formed by intramolecular interaction of the N-terminal half of ERK5 with the C-terminal half (Fig. 8A or B). The next challenge is to determine the region responsible for the nuclear export activity of ERK5 and to determine which model is correct.

Our results clearly demonstrate that both the nuclear import and export mechanisms of ERK5 are active without stimulation. Moreover, inhibition of active nuclear export by LMB treatment causes nuclear accumulation of ERK5. Interestingly, LMB treatment causes nuclear accumulation of endogenous ERK5 in all cell lines tested. These results suggest that both expressed and endogenous ERK5 is continuously shuttling between the nucleus and the cytoplasm, although a large fraction of expressed ERK5 localizes in the cytoplasm under unstimulated conditions.

Expressed ERK5 localizes in the cytoplasm in all cell types examined, including C2C12, HeLa, and COS7 cells (data not shown). Although our proposed mechanism is mostly based on experiments using transfected ERK5 constructs in COS7 cells, essentially the same results were obtained with C2C12 cells (data not shown). Therefore, our mechanism would hold true for expressed ERK5 in all cell types. On the other hand, subcellular localization of endogenous ERK5 is dependent on the cell type. While endogenous ERK5 localizes in the cytoplasm in C2C12 cells, it localizes mostly in the nucleus in COS7 and HeLa cells. In response to stimulation, endogenous ERK5 translocates to the nucleus in C2C12 cells; its nuclear accumulation is enhanced in HeLa cells. Moreover, LMB treatment induces nuclear localization of endogenous ERK5 in C2C12 cells and enhances its nuclear accumulation in COS7 and HeLa cells, suggesting that most or part of endogenous ERK5 localizes in the cytoplasm, due to its nuclear export activity in these cells. Thus, it is likely that subcellular localization of endogenous ERK5 in COS7 and HeLa cells is also regulated partly by our proposed mechanism. It should be pointed out, however, that our proposed mechanism is not a sole mechanism for regulation of subcellular localization of ERK5. In fact, a previous study has shown a nuclear anchoring mechanism of ERK5 (29).

As ERK5 should transmit extracellular signals to proper regions or compartments in cells, regulation of its subcellular localization is important for the regulation of fidelity and specificity of ERK5 signaling. Expressed ERK5 translocates to the nucleus in response to stimulation with natural ligands or by MEK5D expression. Nuclear translocation of endogenous ERK5 takes place in many processes, such as FGF-treated C2C12 cells, neuregulin-treated MCF7 cells, and neurotrophin-treated sensory neurons (14, 37). Moreover, while many targets of ERK5 are transcriptional factors in the nucleus, several direct targets of ERK5 include cytosolic proteins, such as serum- and glucocorticoid-inducible kinase and connexin-43 (4, 18). Thus, control of nucleocytoplasmic shuttling of ERK5 may be important for its function. Our results in this study delineate the molecular mechanism regulating nucleocytoplasmic trafficking of ERK5. While the function of the C-terminal half of ERK5 has been largely unknown, our results, by showing that the C-terminal half has an NLS and is involved in the nuclear export activity of ERK5, suggest that the C-terminal half of ERK5 functions as a regulatory domain for its spatial control.

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