

The Unique C-terminal Tail of the Mitogen-activated Protein Kinase ERK5 Regulates Its Activation and Nuclear Shuttling*

Received for publication, November 8, 2004

Published, JBC Papers in Press, November 17, 2004, DOI 10.1074/jbc.M412599200

Marcus Buschbeck‡ and Axel Ullrich§

From the Max-Planck-Institute of Biochemistry, Department of Molecular Biology, D-82152 Martinsried, Germany

ERK5 is unique among mitogen-activated protein kinases (MAPKs) in that it contains a large C-terminal tail. We addressed the question of how this tail could affect the signaling capacity of ERK5. Gradual deletion of the C-terminal domains resulted in a drastic increase of ERK5 kinase activity, which was dependent on the upstream MAPK cascade, thus indicating a possible auto-inhibitory function of the tail. It is interesting that ERK5 was able to autophosphorylate its own tail. Moreover, ERK5, which was found to be expressed in virtually all kinds of cell lines, localized to nuclear as well as cytoplasmic compartments. The localization of ERK5 was determined by its C-terminal domains, which were also required for appropriate nucleocytoplasmic shuttling. Taken together, these results indicate that ERK5 signaling is directed by the presence of its unique C-terminal tail, which might be the key to understanding the key role of ERK5 in MAPK signaling.

Mitogen-activated protein kinases (MAPKs)¹ are evolutionarily conserved enzymes found in virtually all eukaryotes (1). Although MAPKs often serve as common conduit for a myriad of diverse stimuli, they still lead to highly defined and distinct cellular responses. Recent research has shed some light on this apparently paradoxical situation. The duration and magnitude of MAPK activation on one hand and the localization of MAPKs on the other hand were identified as critical parameters that define specific cellular responses (2, 3). One of the most explored functions of MAPK signaling is the regulation of gene expression by direct or indirect phosphorylation and subsequent activation of transcription factors (4). Because many of these transcription factors are located in the nucleus, translocation of MAPKs to the nuclear compartment is essential for exerting transcriptional control (4, 5).

MAPKs can be grouped into four main subfamilies: the extracellular signal-regulated kinases (ERKs) 1 and 2, the c-Jun N-terminal kinases, p38 stress-activated protein kinases, and

ERK5. The members of the first three subgroups are highly homologous and have overlapping, if not redundant, signaling capabilities. ERK5, however, is the only member of the last and fourth subgroup of MAPKs. The outstanding role of ERK5 within MAPK signaling is best demonstrated by the fact that ERK5-deficient embryos die between days 9.5 and 11.5 because of severe cardiovascular defects (6, 7), whereas mice lacking ERK1 develop normally (8, 9). Using a conditional knock-out, the role of ERK5 could be extended to the maintenance of the cardiovascular system in adult mice (10).

ERK5 differs considerably from other MAPKs, which are only slightly larger than the minimum kinase core defined by cyclin-dependent kinases (11), in that ERK5 contains an unusually long carboxyl-terminal tail. Because of this unique 400-amino acid extension, ERK5 was also termed big MAP kinase 1. The C-terminal tail of ERK5, containing two proline-rich (PR) domains, shares no higher homology with other mammalian proteins. As early as 1995, after the simultaneous cloning of ERK5 by two different groups (12, 13), Zhou and colleagues (13) speculated that the strikingly large tail of ERK5 might contribute to the regulation of its activity and/or localization. Elucidation of the function of the C terminus was also expected to be a key step toward understanding the particular role of ERK5 among MAPKs in the regulation of cell physiology. Although ERK5 has been shown to be involved in the control of various cellular processes ranging from myoblast differentiation and cancer cell proliferation to survival of neurons (14–16), the exact function of its unique C-terminal domains remained largely unknown. The demonstration that the tail of ERK5 could act as transcriptional co-activator further added to the enigmatic character of this big MAPK (17). In this study, we addressed the question of whether and how the unique C-terminal domains could influence the signaling capacity of ERK5. Using sequentially truncated mutants of ERK5, we showed that the C-terminal tail regulated its activation, autophosphorylation, localization, and the dynamics of nucleocytoplasmic shuttling. In agreement with *in vitro* studies performed by Mody *et al.* (18), we found that ERK5 is able to phosphorylate its own C-terminal tail at several sites. Based on our data and results from Yan *et al.* (19) identifying a cryptic nuclear localization sequence in tail region, we propose a hypothetical model that could explain the behavior of ERK5 mutants and prove helpful when addressing questions of ERK5 function. In this model, we consider ERK5 to exist in a closed or open confirmation, with the latter stabilized by activating phosphorylations and allowing nuclear import.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—For the generation of ERK5 antibodies, rabbits were immunized with a fusion protein of GST and ERK5 amino acids (aa) 410–558. The anti-HA antibody 12CA5 (Roche Diagnostics) was used for immunoprecipitation and the HA.11 (BAbCO) for Western blot analysis. Anti-ERK2 K23 antibody was from Santa Cruz Biotechnology and anti-tubulin antibody was from Sigma-Aldrich. Anti-Ran

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Center for Genomic Regulation (CRG), Passeig Maritim 37-49, E-08003 Barcelona, Spain. E-mail: marcus.buschbeck@crg.es

§ To whom correspondence should be addressed: Max-Planck-Institute of Biochemistry, Dept. of Molecular Biology, Am Klopferspitz 18a, D-82152 Martinsried, Germany. Tel.: 49-89-8578-2512; Fax: 49-89-8578-2454; E-mail: ullrich@biochem.mpg.de.

¹ The abbreviations used are: MAPK, mitogen-activated protein kinase; aa, amino acids; CL, crude cell lysate; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GSH, glutathione; GST, glutathione S-transferase; HA, hemagglutinin; IP, immunoprecipitation; NES, nuclear export sequence; NLS, nuclear localization sequence; WB, Western blot.

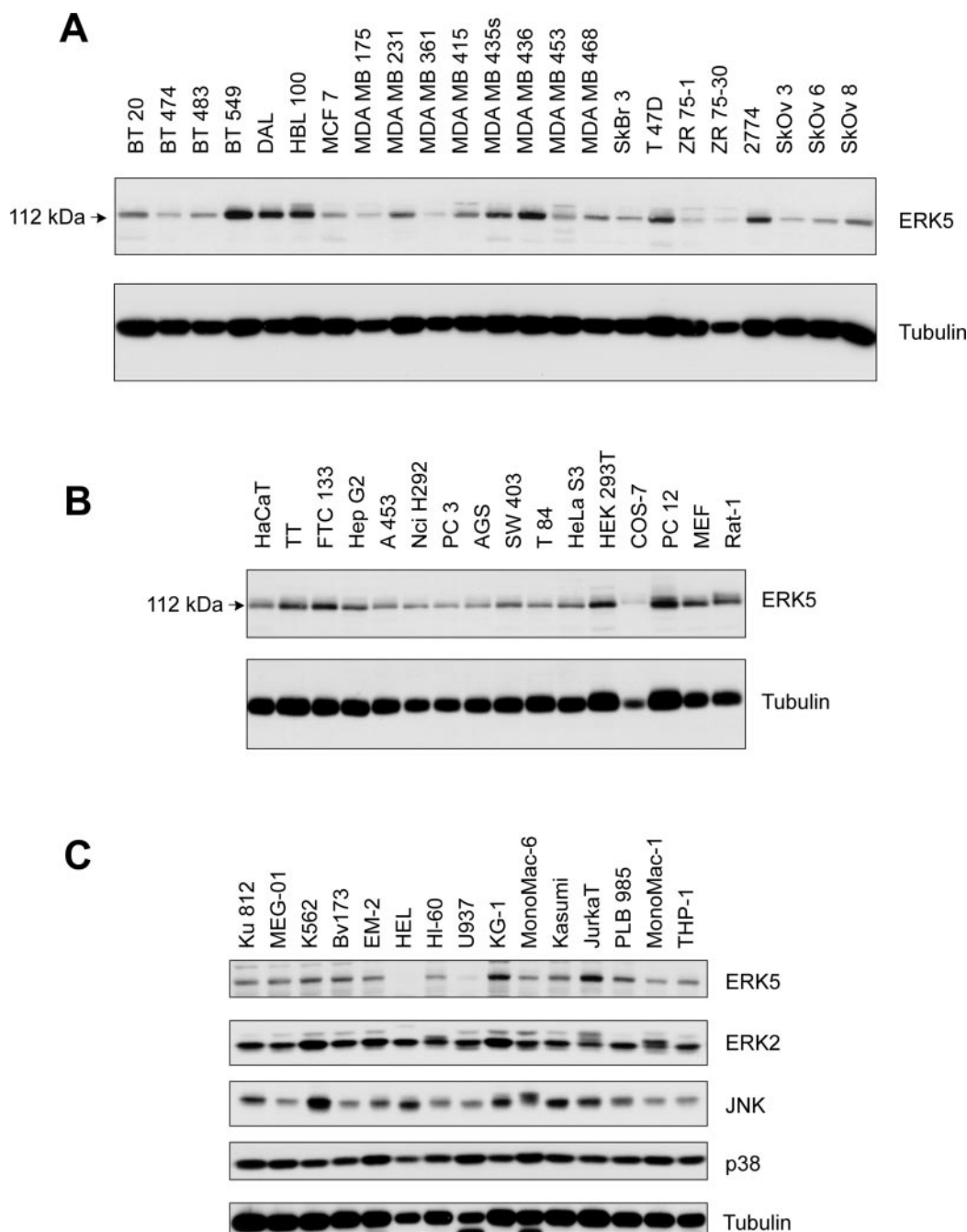


FIG. 1. **Expression of ERK5 in various cell lines.** A, anti-ERK5 immunoprecipitates from crude cell lysates of 19 breast and 4 ovarian cancer cell lines (*top*) and 5% of input lysate (*bottom*) were analyzed by anti-ERK5 and anti-tubulin immunoblots, respectively. B, several human cancer and non-cancer cell lines, COS-7 cells, and three rodent cell lines were analyzed as described in A. C, expression of different MAPKs in leukemia cell lines was compared by immunoblot analysis of crude cell lysates using anti-ERK5, ERK2, c-Jun N-terminal kinase, p38, and tubulin antibodies.

GAP antibody was kindly provided by Frauke Melchior (Max-Planck-Institute of Biochemistry, Martinsried, Germany). Horseradish peroxidase-conjugated goat anti-rabbit antibody was from Bio-Rad, goat anti-mouse antibody was from Sigma, and donkey anti-goat antibody was from Jackson ImmunoResearch Laboratories. Chemiluminescence reagents and [γ - 32 P]ATP (6000 Ci/mmol) were from PerkinElmer Life and Analytical Sciences. Protein A, protein G, and GSH-Sepharose beads were purchased from Amersham Biosciences. Epidermal growth factor was from Invitrogen. Leptomycin A and the MAPK inhibitor U0126 were purchased from Calbiochem/Merck Biosciences. Nitrocellulose membrane was from Schleicher & Schüll. All other reagents were obtained from Merck.

Plasmid Construction and Protein Purification—All cDNAs used for transient transfection were in cytomegalovirus promoter-driven expression plasmid pcDNA3 (Invitrogen); HA-ERK2 (20), HA-ERK5, and MEK5 DD (21). Constructs for truncated variants of ERK5 and the ERK2/ERK5 chimera consisting of aa 410–815 of ERK5 C-terminally

fused to full-length ERK2 were generated by standard PCR techniques. The non-activatable form of ERK5 was generated by site-directed mutagenesis of the activating motif TEY to AEF. Fusion proteins of GST and portions of ERK5 were constructed by subcloning cDNA fragments encoding ERK5 aa 410–558, 558–709, and 710–815 into pGEX5X vectors (Amersham Biosciences). Bacterial expression construct for GST-MEF2C (aa 175–327) was kindly provided by Silvio Gutkind (Bethesda, MD). All GST fusion proteins were expressed in BL21 DE3 Codon + (Stratagene) and purified with glutathione-Sepharose beads. Sequences of used oligonucleotides are available on request.

Cell Culture and Transfection—All cells with the exception of PC-12 (P. Cohen, Dundee, Scotland, UK), HaCaT (K. Specht, Martinsried, Germany), MEF (L. Hennighausen, Bethesda, MD) and DAL (G. Natali, Rome, Italy) were obtained from either Deutsche Sammlung von Mikroorganismen und Zellkulturen, the European Collection of Cell Cultures, or the American Type Culture Collection and were cultivated following the supplier's instructions. Cell culture reagents were pur-

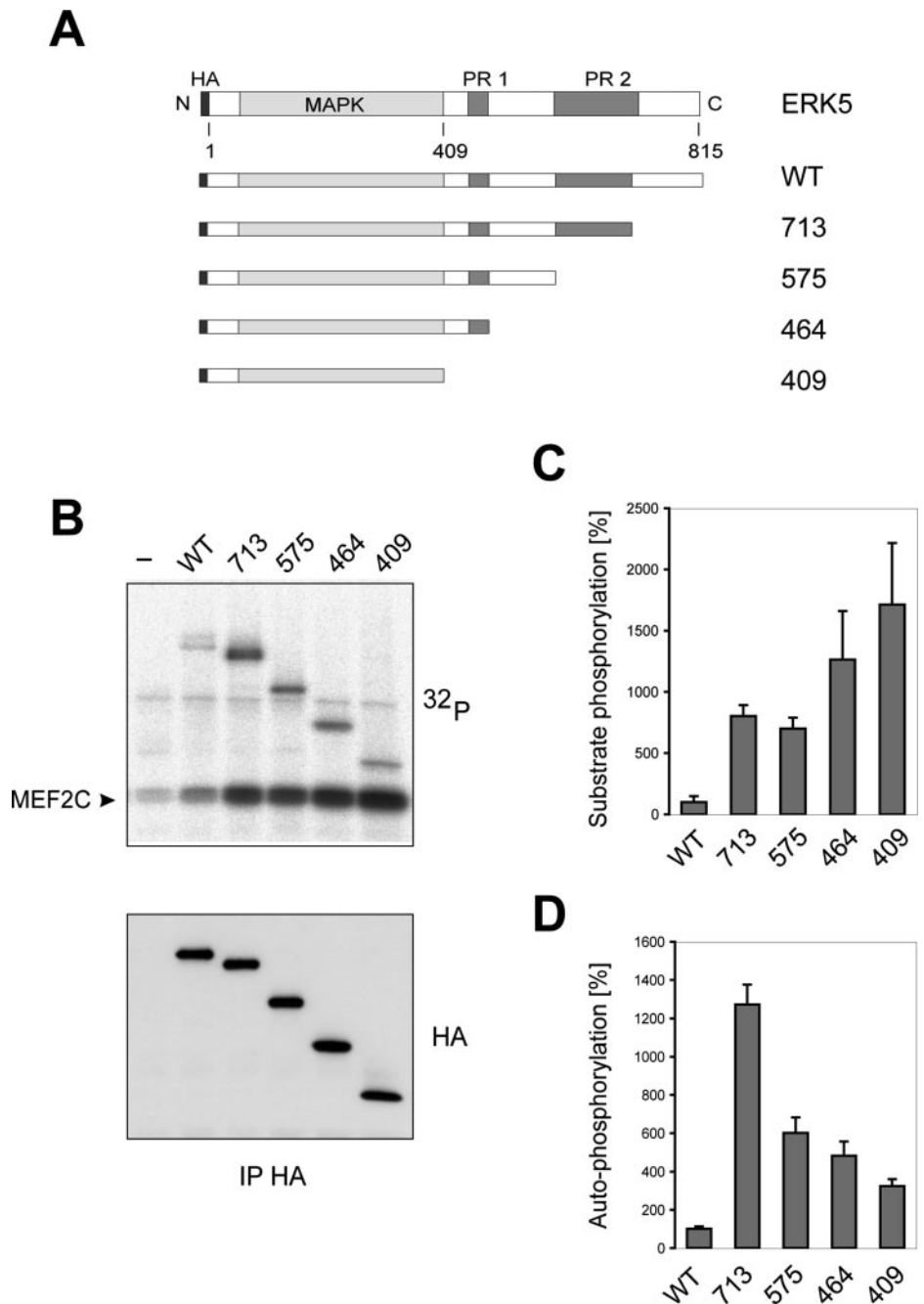


FIG. 2. C-terminal domains reduce activity of ERK5. COS-7 cells were transfected with expression constructs for HA-tagged ERK5 and several truncated mutants. *A*, a schematic scheme of used constructs is shown. Numbers given as names for truncated mutants refer to the last amino acid. *B*, cells were lysed 48 h after transfection, and exogenous ERK5 variants were immunoprecipitated using anti-HA antibody. Precipitates were subjected to kinase reactions using GST-MEF2C as substrate. Samples were separated and analyzed by anti-HA immunoblot and autoradiography (*top* and *bottom*, respectively). *IP*, immunoprecipitation. *C*, a quantification of kinase activity is shown. Substrate phosphorylation was corrected for the amount of precipitated kinase and normalized. Data represent the mean of three independent experiments \pm S.D. *D*, a quantification of autophosphorylation is shown. Data represent the mean of three independent experiments \pm S.D.

chased from Invitrogen. COS-7 cells were plated on 6- or 12-well dishes and transfected with 1 or 0.3 μ g of DNA, respectively, following Qiagen's Polyfect protocol. Cells were generally processed and analyzed after 48 h. If required, cells were starved by serum withdrawal for 24 h.

Lysis, Immunoprecipitation, and Immunoblot Analysis—Cell cultures were washed with PBS and lysed with lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerine, and 1% Triton X-100) supplemented with phosphatase and protease inhibitors (10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0.5% aprotinin). Cellular debris was removed by centrifugation. Protein concentrations were determined using the Micro BCA protein assay kit (Pierce Chemical). Supernatants were pre-cleared with 20 μ l of Sepharose slurry. Immunoprecipitations were carried out as described previously with slight modifications (21). In brief, lysates were incubated with anti-HA or anti-ERK5 antibodies together with 20 μ l of either protein G or A-Sepharose, respectively, for at least 3 h on a rotating wheel at 4 $^{\circ}$ C. Precipitates were washed three times with lysis buffer, suspended in 1.2 \times SDS sample buffer, boiled for 3 min, and subjected to gel electrophoresis. Proteins were transferred to nitrocellulose membranes and immunoblotted. If quantification was necessary, filters were exposed to the LAS1000 chemiluminescence

camera (Fujifilm) and analyzed with the program Image Gauge 3.3 (Fujifilm).

Kinase Assays—For *in vitro* ERK5 kinase assays, immunoprecipitates were washed twice with lysis buffer and once with kinase assay buffer (25 mM HEPES, pH 7.5, 20 mM MgCl_2 , 2 mM dithiothreitol, and 0.5 mM orthovanadate). Samples were suspended in 30 μ l of kinase assay buffer containing 50 μ M ATP, 1.5 μ Ci of [γ - 32 P]ATP, and 3 μ g of GST-MEF2C as substrate and incubated for 20 min at 30 $^{\circ}$ C under constant shaking. For the measurement of ERK2 and ERK2/5 kinase activity, reactions were performed in a different kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl_2 , 1 mM dithiothreitol, and 0.5 mM orthovanadate) containing 15 μ M ATP, 1.5 μ Ci of [γ - 32 P]ATP, and 10 μ g of myelin basic protein (MBP) as substrate for 10 min at room temperature. In general, kinase reactions were stopped by the addition of 2 \times SDS sample buffer and boiling. Samples were resolved on SDS-PAGE and transferred to nitrocellulose. Phosphorylation was detected by filmless autoradiographic analysis using the BAS2500 Reader (Fujifilm) and quantified with Image Gauge 3.3 (Fujifilm). The amount of precipitated kinase was visualized by immunoblot analysis.

Immunofluorescence—COS-7 cells were seeded at 2×10^4 cells/cm 2 on glass coverslips. Transfections were performed as described above,

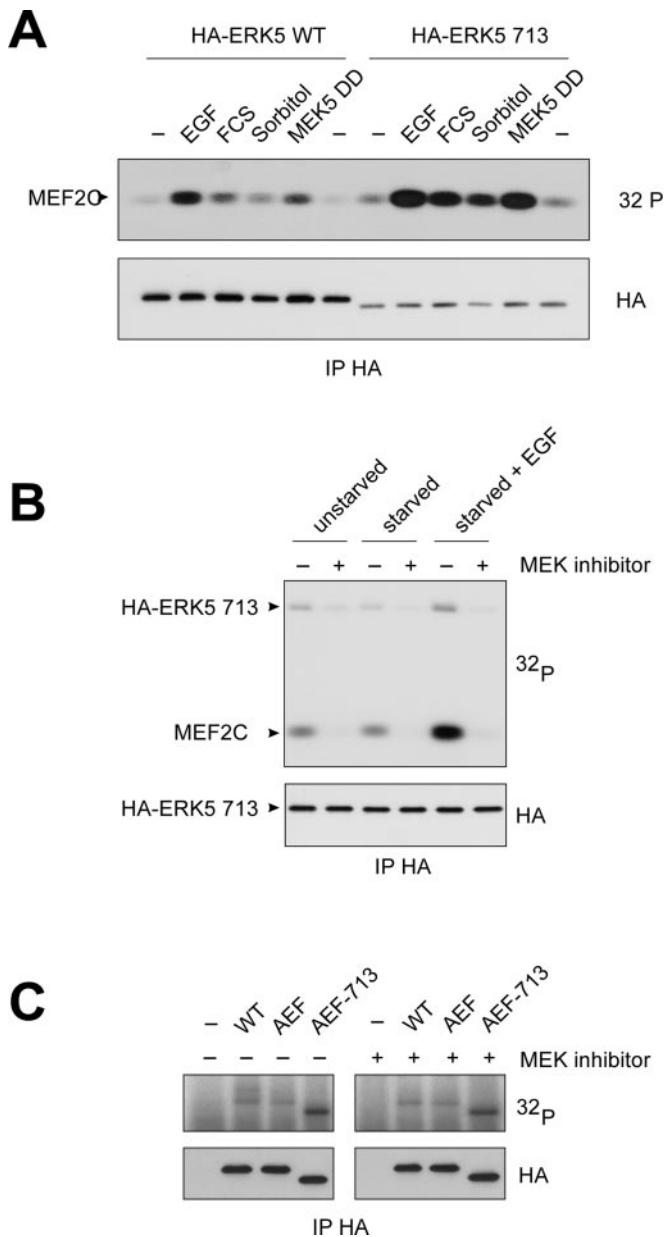


FIG. 3. C-terminal deletion facilitates activation. *A*, transfected COS-7 cells were serum-starved overnight and stimulated as indicated. After lysis, anti-HA immunoprecipitates were subjected to immunocomplex kinase assays using GST-MEF2C as substrate. After separation, samples were analyzed by anti-HA immunoblot and autoradiography. *IP*, immunoprecipitation. *B*, COS-7 cells overexpressing HA-tagged ERK5 713 were either left untreated or were starved by serum withdrawal overnight, pretreated with a 10 μ M concentration of the MEK inhibitor U0126 for 30 min, and stimulated with 20 ng/ml epidermal growth factor for 15 min as indicated. Anti-HA immunoprecipitates were subjected to *in vitro* kinase reactions using MEF2C as substrate. After separation, samples were analyzed by autoradiography and anti-HA immunoblot. *C*, transfected COS-7 cells were pre-treated with U0126 for 1 h if indicated. HA-ERK5 wild-type (WT), non-activatable AEF, and truncated AEF-713 were precipitated with anti-HA antibody and subjected to immunocomplex kinase reactions. Autophosphorylation was monitored by autoradiography, and the amount of precipitated kinase by anti-HA immunoblot.

and cells were processed for immunofluorescence after 48 h of further culture. Cells were washed twice with PBS and fixed with 3.7% paraformaldehyde for 20 min at room temperature. Samples were washed again and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After washing thoroughly, samples were incubated in pure fetal calf serum for at least 1 h. After washing twice with PBS containing 0.05% Tween 20 samples were incubated with a 1:1000 dilution of anti-HA or anti-ERK5 antibodies in PBS/0.05% Tween 20 and fetal calf

serum (1:1) for 1 h. After three more washes with PBS/0.05% Tween 20, cells were incubated with respective secondary Cy2-labeled goat antibodies (Jackson ImmunoResearch Laboratories) in a 1:1000 dilution for 1 h. Samples were washed three more times with PBS/0.05% Tween 20, rinsed in distilled water, and mounted. DNA was stained for 10 min with 1 μ g/ml bisbenzamide, H33342, which was included in the penultimate washing step.

Preparation of Nuclear Extracts—48 h after transfection 1×10^6 COS-7 cells were trypsinated and collected by centrifugation at $500 \times g$. After washing twice with PBS cells were lysed in hypotonic lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40) supplemented with phosphatase and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM NaF, and 0.5% aprotinin) on ice for 5 min. Nuclei were precipitated by centrifugation at $500 \times g$, and supernatants were kept as cytosolic extracts. Nuclei pellets were loosened by vortexing, washed once with hypotonic lysis buffer, and finally dissolved in radioimmunoprecipitation assay buffer (20 mM Tris, pH 7.5, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, and 150 mM NaCl) supplemented with phosphatase and protease inhibitors. Nuclear extracts were sonified (10 min at 4 °C in a sonification bath) to destroy genomic DNA. Cellular debris were removed from both extracts by centrifugation. After determining protein concentration extracts were analyzed by immunoblotting.

RESULTS

Expression Pattern of ERK5—We set out to analyze ERK5 expression in a large array of cell lines. Crude cell lysates and anti-ERK5 immunoprecipitates from various human and non-human cancer and non-cancer cell lines were analyzed by anti-ERK5 immunoblot. Because several lines of investigation pointed toward a role for ERK5 in breast cancer, attributing either survival or proliferative potential to ERK5 (15, 22, 23), we included 19 established breast cancer cell lines in our analysis. As shown in Fig. 1*A*, a band migrating at 112 kDa corresponding to ERK5 was readily detected in all 19 breast cancer lines as well as in four ovarian cancer cell lines analyzed. Extending the analysis to a wider variety of cell lines, we found ERK5 to be expressed in virtually all cell lines tested (Fig. 1, *B* and *C*) albeit at different levels. The resulting expression profile of ERK5 did not correlate with those of other MAPKs (ERK2, c-Jun N-terminal kinase, or p38) in cell lines derived from the hematopoietic system (Fig. 1*C*). The presence of ERK5 in such a wide variety of cell types suggests that ERK5 is most probably ubiquitously expressed and gives no hint of a particular cell type-specific function.

C-terminal Deletions Result in Enhanced Kinase Activity—Next, we tested whether sequential truncation of the tail would influence the enzymatic activity of ERK5. Therefore, we transfected expression constructs for hemagglutinin (HA)-tagged wild-type ERK5 and several truncated mutants into COS-7 cells (a schematic representation of the mutants used is shown in Fig. 2*A*). Kinase activity of exogenous ERK5 in untreated cells was analyzed in anti-HA immunocomplex kinase assays using a fusion protein of GST and a portion of the transcription factor MEF2C as substrate. As shown in Fig. 2, *B* and *C*, deletion of the last 100 amino acids in the 713 mutant of ERK5 resulted in a drastic increase in kinase activity. Deleting the PR2 domain did not further add to the effect on kinase activity, whereas two mutants, ERK5 464 and 409, lacking also the linker region between the two PR domains and the PR1 itself, were even more active. Increased kinase activity of truncated ERK5 was also indicated by enhanced autophosphorylation (Fig. 2, *B* and *D*).

The strong influence of C-terminal truncations on the activity of ERK5 in untreated cells led us to question whether the observed increase in the activity of ERK5 would be dependent on its specific upstream kinase MEK5. Therefore, we tested whether stimulation of starved cells with epidermal growth factor, fetal calf serum, or sorbitol, three known activators of the MEK5-ERK5 pathway (21, 24–26), would be able to enhance the activity of mutant ERK5. As shown in Fig. 3*A*, the

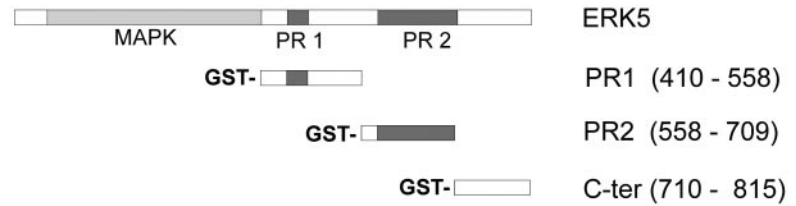
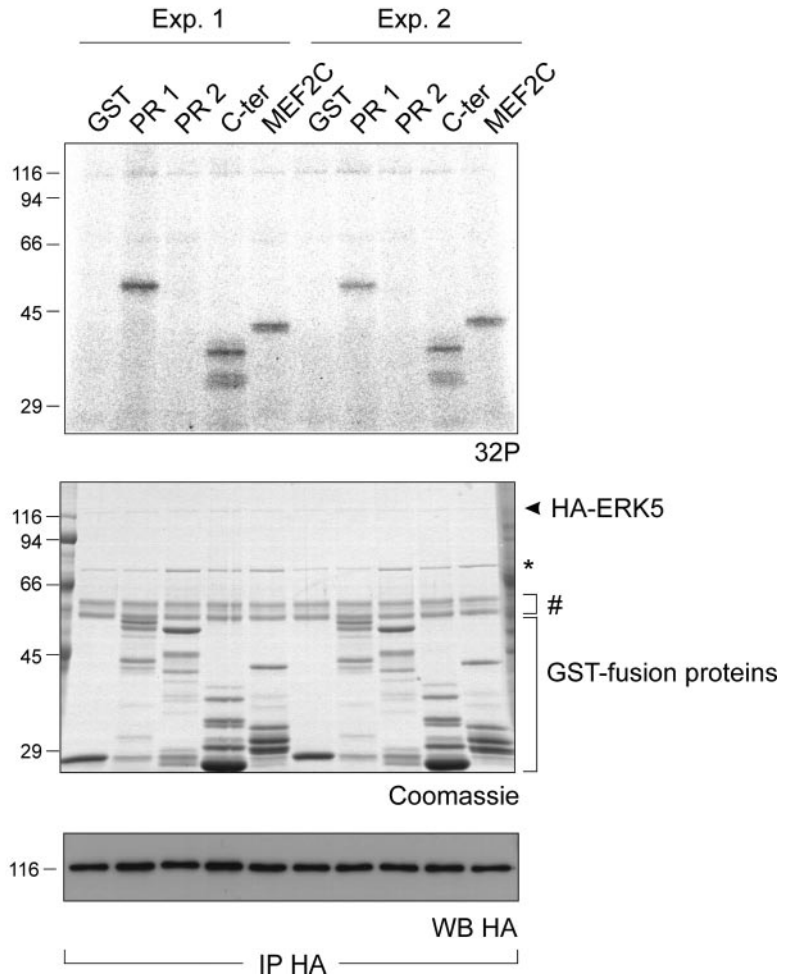
A**B**

FIG. 4. ERK5 phosphorylates its C-terminal domains. The ability of ERK5 to phosphorylate fusion proteins of GST and different portions of the C-terminal tail of ERK5 was tested in kinase reactions *in vitro*. **A**, schematic representation of the used fusion proteins. **B**, HA-ERK5 was immunoprecipitated from lysates of transfected COS-7 cells. Samples were subjected to kinase reactions containing different GST fusion proteins. Samples were split, separated, and analyzed by autoradiography, Coomassie staining, and anti-HA immunoblot (*top*, *middle*, and *bottom*, respectively). *IP*, immunoprecipitation; *WB*, Western blot.

activity of ERK5 713 was indeed potently induced in cells treated with either stimulus, as was the case for wild-type ERK5. Likewise, co-expression of a constitutively active MEK5 S311D,T315D (DD) also led to an increase in activity of wild-type and truncated ERK5. The 713 mutant also retained sensitivity to interference with the activating kinase cascade. As shown in Fig. 3B, activity of ERK5 713 in untreated as well as in epidermal growth factor-stimulated cells was blocked by pre-treating cells with a MEK inhibitor. These results indicate that C-terminal truncations of ERK5 most probably facilitate its activation by the MAPK cascade containing MEK5. AEF mutants of ERK5 lacking the two phosphorylation sites for MEK5 cannot be activated but possess basal activity.² As

shown in Fig. 3C, deletion of the last 100 amino acids of such a mutant (AEF-713) resulted in an increase of ERK5 phosphorylation in immunocomplex kinase assays in a MEK-independent manner, thus indicating that the far C terminus might also impair autophosphorylation of non-activated ERK5.

ERK5 Autophosphorylates on Its C-terminal Tail—The capacity of ERK5 to auto-phosphorylate has been used in the past to monitor ERK5 activity (21, 27, 28). Thus, the enhanced activity of the 713 mutant was not only reflected by increased phosphorylation of the substrate MEF2C but also by its autophosphorylation (Figs. 2, B and C, and 3B). Although further truncated mutants were either similar or even more active than ERK5 713 as indicated by substrate phosphorylation, the degree of autophosphorylation decreased with shortening of the tail. Therefore, we tested whether ERK5 would be able to phosphorylate fusion proteins of different portions of its C-

² Buschbeck, M., Hofbauer, S., Di Croce, L., Keri, G., and Ullrich, A. (2004) *EMBO Rep.*, in press.

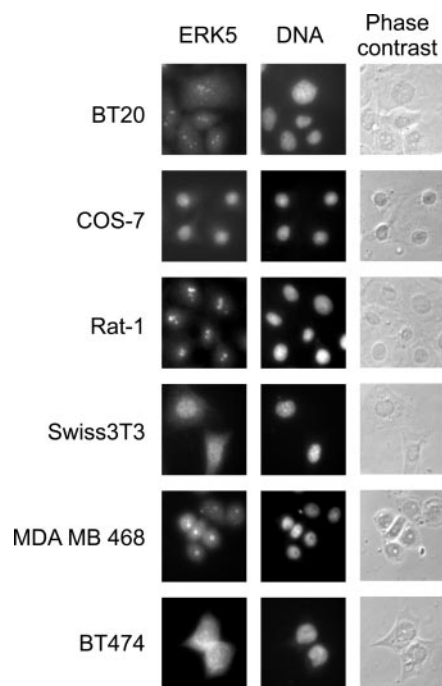


FIG. 5. **ERK5 localizes to cytosol and nuclei in different cell lines.** Human BT474, BT20, and MDA MB 468, avian COS-7, and rodent Rat-1 and Swiss 3T3 were fixed with paraformaldehyde and permeabilized with detergent. Localization of cellular ERK5 was analyzed by immunofluorescence analysis using anti-ERK5 antibody (*left*), and DNA was stained with bisbenzimid (*middle*). Phase contrast images of cells are shown on the *right*. Each horizontal panel shows identical sections of cells.

terminal tail and GST. These fusion proteins contained either PR1 domain, PR2, or the C-terminal 100 amino acids (Fig. 4A). As shown in Fig. 4B, immunoprecipitated ERK5 phosphorylated the fusion proteins containing either the PR1 or the far C terminus to an extent similar to that of MEF2C but not the fusion protein of PR2 or GST itself. These results are in accordance with *in vitro* results from the group of Phil Cohen analyzing autophosphorylation of purified ERK5 *in vitro*. The tail was found to contain five of six main autophosphorylation sites; three of these sites are located in the fusion protein containing PR1, and the other two are in the far C-terminal domain (18). Although these results establish that ERK5 is indeed phosphorylating its own tail, the consequences of these modifications remain elusive.

Nuclear Localization of ERK5—Many MAPK substrates, which include several transcription factors, reside in the nucleus. Translocation of MAPKs from the cytosolic to the nuclear compartment is therefore essential for their ability to regulate gene transcription (29). Therefore, we analyzed the distribution of ERK5 in different cell lines. We have reported previously that in COS-7 cells, ERK5 localizes not only to the cytosol but also to the nuclear compartment (21). As shown in Fig. 5, nuclear ERK5 staining was not restricted to COS-7 cells but was also found in five other cell lines tested, albeit to different extents. The cellular distribution of ERK5 varied largely between these different cells and ranged from predominant nuclear localization in COS-7 cells to an overall diffuse pattern in BT474 cells. It is interesting that ERK5 was found to concentrate in one or more discrete spots in nuclei of MDA MB 468 and BT20 breast cancer cells as well as in those of Rat-1 fibroblasts.

The C Terminus of ERK5 Is a Determinant of Its Nuclear Localization—Small molecules including most MAPKs can, in principle, enter the nucleus by simple diffusion through the

nuclear pore. In contrast, proteins with a molecular mass greater than around 60 kDa are actively transported from one side of the nuclear envelope to the other (30, 31). Because the nuclear import and export of the considerably larger ERK5 can thus be expected to occur by the latter mechanism, we sought to determine whether its C-terminal domain is involved in the regulation of its nuclear localization. We analyzed the localization of ERK5 and its C-terminally truncated mutants in transfected COS-7 cells by immunofluorescence. Although overexpressed full-length ERK5 was found to mainly reside in the cytosolic compartment, deletion of the last 100 amino acids in the 713 mutant resulted in predominant nuclear localization (Fig. 6, A and B). Similar to the results obtained in the analysis of kinase activity (Fig. 2, B and C), further truncated 575 mutant was phenotypically identical to 713 and was also predominantly nuclear. In contrast, the two shorter 464 and 409 mutants lacking either most or all of the C-terminal tail were similarly distributed over nuclear and cytosolic compartments in the majority of cells. It is noteworthy that Yan *et al.* identified a NLS between amino acids 505 and 539 (19), which is absent in the two shortest mutants. According to the immunofluorescence analysis, wild-type as well as mutant ERK5 were detected in cytosolic extracts by immunoblot analysis. All truncated forms of ERK5 were further readily detected in nuclear extracts. In particular, 713 and 575 were enriched in the nuclear fraction, whereas wild-type ERK5 was hardly detectable at all (Fig. 6C). Having shown before that C-terminal deletions favor the activation of ERK5, we used the truncated, nonactivatable AEF-713 to test whether activation would be required for nuclear translocation. As shown in Fig. 6D, AEF-713 was predominantly cytosolic. Although ERK5 carries its own NLS and would thus be able to enter the nucleus on its own, its translocation is still governed by the activating kinase cascade containing MEK5.

We next sought to determine whether the tail is able to influence the localization of another MAPK as well. Therefore, we generated a chimeric protein by fusing the complete tail of ERK5 to full-length ERK2, the closest homologue of ERK5 (Fig. 7A). The wild-type form of ERK2, which is devoid of any domains besides the conserved MAPK domain, was found to reside in the nucleus as well as in the cytosol of cells. Cells expressing the ERK2/5 chimera displayed a marked increase in cells with predominant nuclear staining (Fig. 7, B and C). It is noteworthy that the subcellular distribution of wild-type ERK2 was similar to the pattern of ERK5 409 mutant, which lacks the entire tail. Because translocation of ERK2 depends on its activation (3), we tested whether ERK2 activity was altered by addition of the tail. In contrast to ERK5, which is strongly inhibited by its own tail, the activity of the chimeric protein ERK2/5 was comparable with wild-type ERK2 (Fig. 7D).

Subcellular distribution of proteins monitored in fixed cells can be considered a snapshot of a highly dynamic process of protein exchange between all compartments of the cell. To gain some information on the dynamics of nuclear import and export of ERK5, we used leptomycin A to block the nuclear export machinery (32) and tested how this would affect the localization of ERK5. Cells expressing either wild-type ERK5 or the 409 mutant were treated with the toxin and subsequently analyzed by immunofluorescence. Treatment of cells with leptomycin A strongly induced nuclear accumulation of wild-type ERK5, whereas 409 mutant was only mildly affected (Fig. 8, A and B).

Taken together, these results suggest a profound role of the unique C-terminal domains of ERK5 in the regulation of its function by influencing its activation, its localization, and the kinetics of its nuclear shuttling.

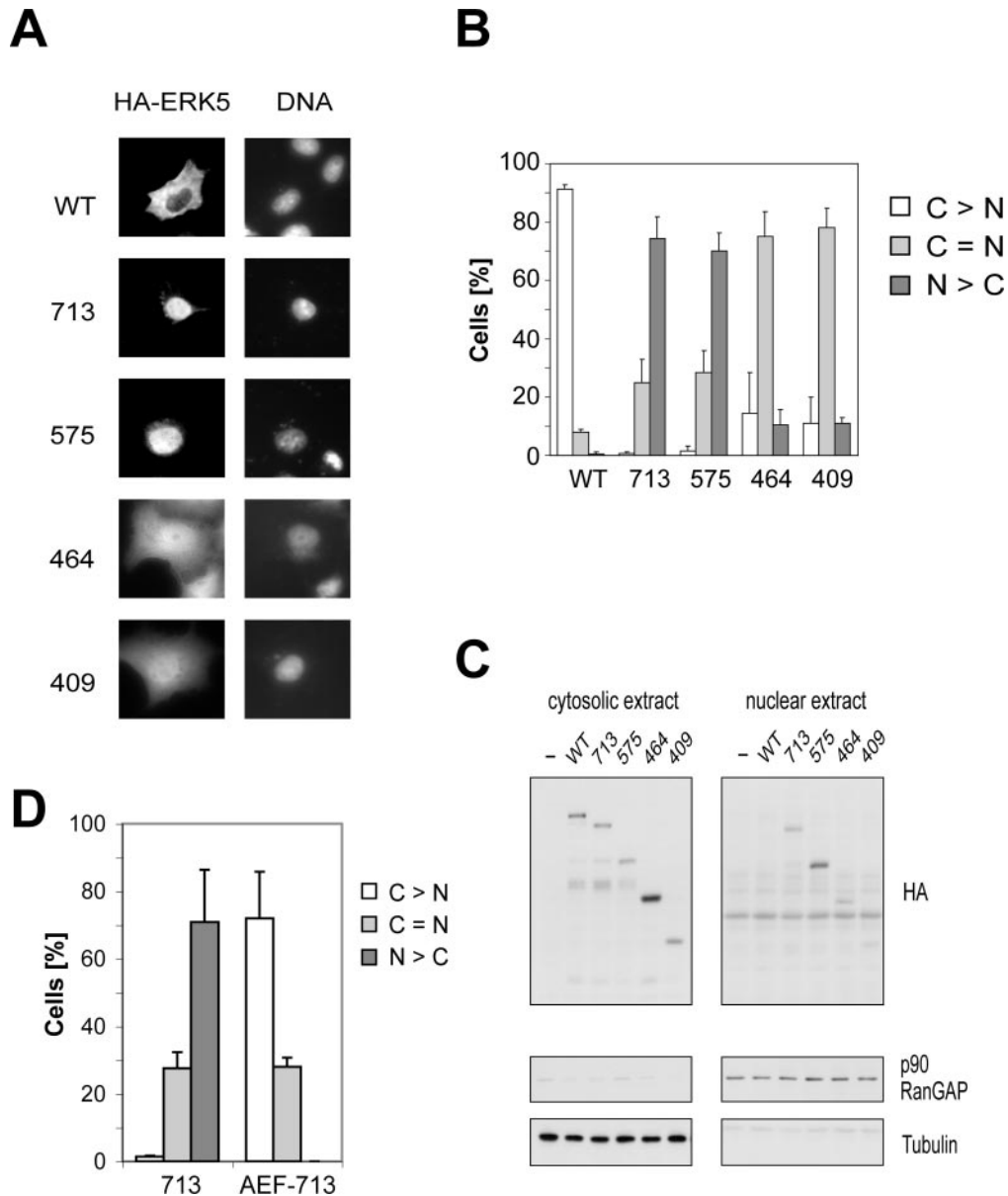


FIG. 6. The C-terminal tail of ERK5 regulates its localization. **A**, COS-7 cells were transiently transfected with expression constructs of wild-type HA-ERK5 or different truncated mutants and analyzed by fluorescence microscopy after 48 h. Exogenous ERK5 was visualized by immunofluorescence using anti-HA antibodies (*left*) and DNA was stained with bisbenzimid (*right*). Each *horizontal panel* shows identical sections of COS-7 cells. **B**, cells were scored according to their ERK5 staining into three categories: uniform distribution between cytoplasm and nucleus ($C = N$), predominantly nuclear ($C < N$), and predominantly cytoplasmic ($C > N$). Approximately 150 cells were scored per coverslip. Data represent the mean of at least two independent experiments \pm S.D. **C**, nuclear and cytosolic extracts from transfected COS-7 cells expressing HA-tagged mutants of ERK5 were analyzed by anti-HA immunoblot. Equal loading was assured by reprobing the membrane with anti-RanGAP and anti-tubulin antibodies. **D**, COS-7 cells were transfected with HA-ERK5 713 and AEF-713 and analyzed as described under **A** and **B**.

DISCUSSION

The C-terminal tail of ERK5 is unique among MAPKs. In this study, we addressed the question of whether this large C-terminal extension could affect the signaling capacity of ERK5. The data presented show that the tail influences not only the activation of ERK5 but also its nucleocytoplasmic shuttling and might thus define the very particular role of ERK5 within the complexity of cellular signal transduction.

Deletion of the last hundred amino acids of ERK5 (713 mutant) led to a marked increase in its kinase activity as assessed by the *in vitro* phosphorylation of the specific substrate MEF2C. But how could the C-terminal tail of ERK5 influence its enzymatic activity? We considered two possibilities: C-terminal truncation could either result in an enhanced basal activity of ERK5 or facilitate its activation by its specific upstream kinase MEK5, resulting in an increase in number of

activated ERK5 molecules. Based on two observations, we favor the latter as the most likely underlying mechanism. Shortened mutants of ERK5 were sensitive not only to stimulation of cells with known activators of ERK5 but also to inhibition of its activating kinase MEK5. Others have speculated previously that the tail could inhibit kinase activity (33). Such an autoinhibition could occur, for instance, if ERK5 adopted a conformation in which the tail masked the conserved MAPK docking motif that mediates the interaction of MAPKs with their activation MEKs (34). Self-inhibition by an intramolecular mechanism is a common theme in the regulation of kinase activity and has been extensively studied in the case of the tyrosine kinase c-Src (35) and has more recently been demonstrated for tyrosine kinases c-Abl and Bcr-Abl (36, 37).

Nucleocytoplasmic transport of signaling molecules is essential for the regulation of eukaryotic cellular processes such as

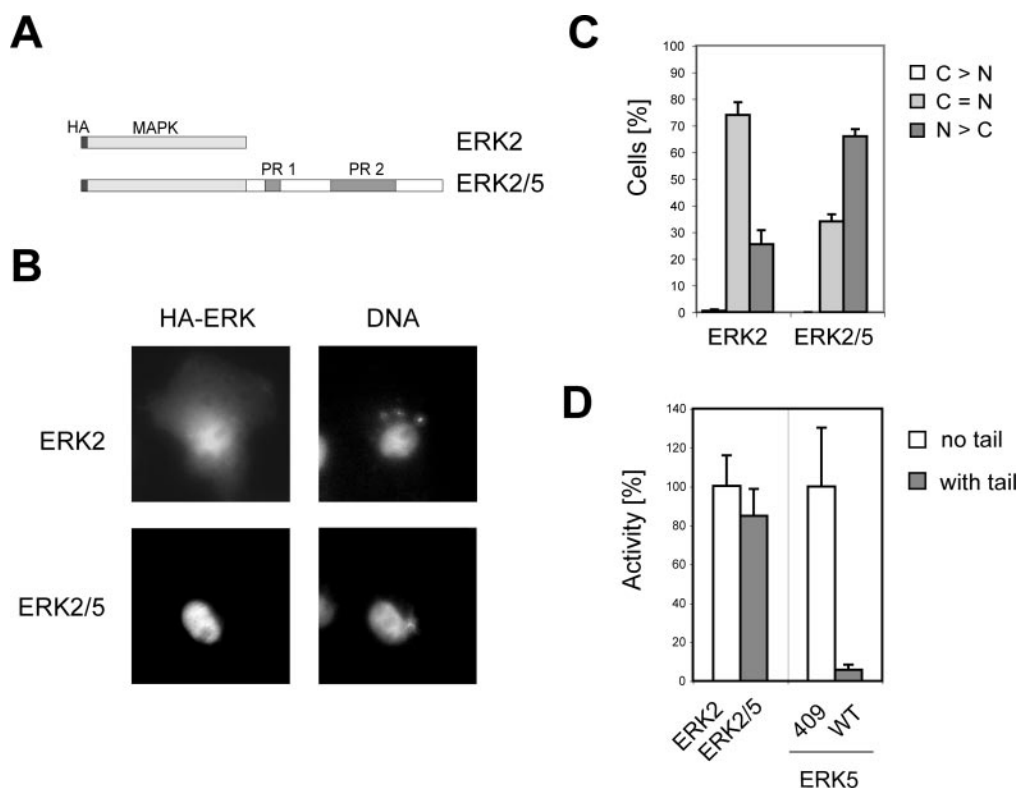


FIG. 7. Enhanced nuclear localization of chimeric ERK2/5. *A*, schematic representation of ERK2 and chimeric ERK2/5. *B*, COS-7 cells were transiently transfected with pcDNA3 ERK2 and ERK2/5 and analyzed by fluorescence microscopy after 48 h. ERK5 was visualized by immunofluorescence using anti-ERK5 antibodies (*left*) and DNA was stained with bisbenzimid (*right*). *C*, cells were scored according to their ERK5 staining into three categories: uniform distribution between cytoplasm and nucleus ($C = N$), predominantly nuclear ($C < N$), and predominantly cytoplasmic ($C > N$). *D*, HA-tagged ERK2, ERK2/5, ERK5 wild-type (WT), and 409 mutant were immunoprecipitated from transfected COS-7 cells and subjected to *in vitro* kinase assays. Myelin basic protein and GST-MEF2C were used as substrate for ERK2 and ERK5, respectively. Data represent the mean of two independent experiments \pm S.D.

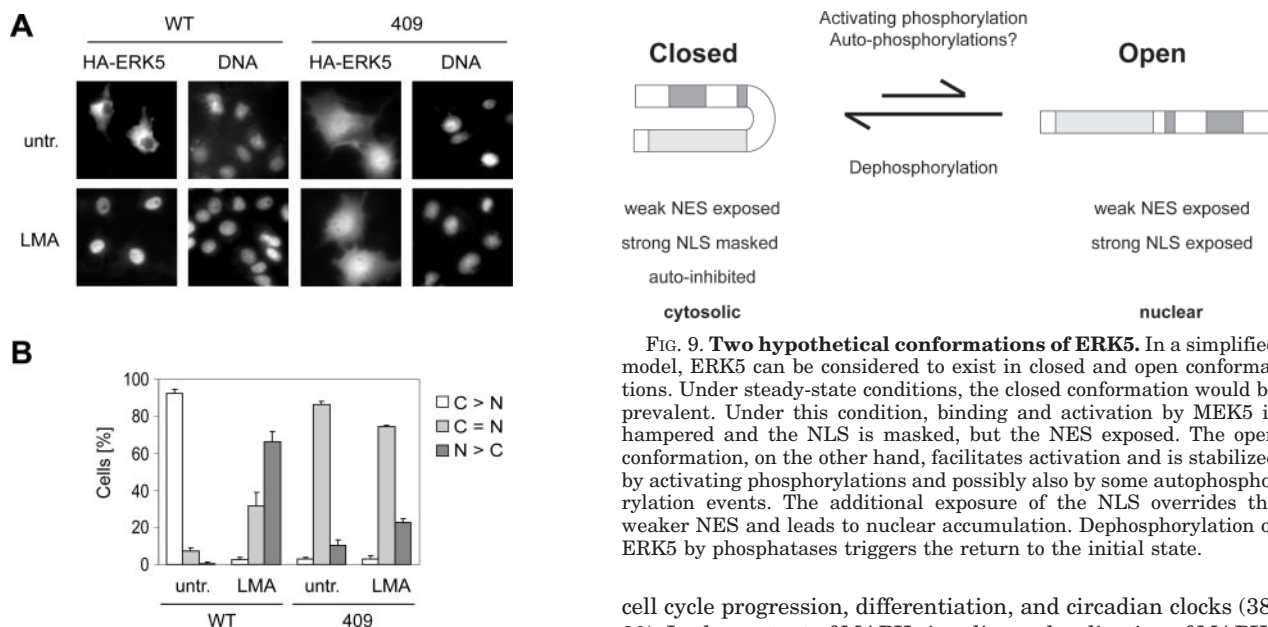


FIG. 8. The C terminus is required for highly dynamic nuclear shuttling of ERK5. *A*, transfected COS-7 cells expressing either wild-type HA-ERK5 or ERK5 409 mutant lacking the complete C-terminal tail were treated with 6 ng/ml of the nuclear export inhibitor leptomycin A (LMA) or left untreated (*untr.*). After 3 h, cells were fixed and analyzed by anti-HA immunofluorescence (*left*). DNA was visualized by staining with bisbenzimid (*right*). *B*, cells were scored according to their ERK5 staining into three categories: uniform distribution between cytoplasm and nucleus ($C = N$), predominantly nuclear ($C < N$), and predominantly cytoplasmic ($C > N$).

FIG. 9. Two hypothetical conformations of ERK5. In a simplified model, ERK5 can be considered to exist in closed and open conformations. Under steady-state conditions, the closed conformation would be prevalent. Under this condition, binding and activation by MEK5 is hampered and the NLS is masked, but the NES exposed. The open conformation, on the other hand, facilitates activation and is stabilized by activating phosphorylations and possibly also by some autophosphorylation events. The additional exposure of the NLS overrides the weaker NES and leads to nuclear accumulation. Dephosphorylation of ERK5 by phosphatases triggers the return to the initial state.

cell cycle progression, differentiation, and circadian clocks (38, 39). In the context of MAPK signaling, relocalization of MAPKs from the cytosol to the nucleus is required for phosphorylating and activating those transcription factors that exclusively reside in nuclear compartments (3, 29). The analysis of several cell lines indicated that endogenous ERK5 localized to cytoplasmic as well as nuclear compartments. When overexpressed in COS-7 cells, ERK5-specific staining was predominantly cytoplasmic; however, deletion of the last hundred amino acids resulted in its nuclear accumulation. Further subsequent de-

letion of most or all of the ERK5 tail resulted in a loss of its predominant nuclear localization and in equal redistribution over cytosol and nucleus. Nuclear localization of truncated ERK5 was MEK5-dependent because mutation of the two phosphorylation sites for MEK5 resulted in relocalization of ERK5 to the cytosolic compartment. The distribution of the mutants can be explained by the presence of functional nuclear import and export sequences in the tail region. Yan *et al.* (19) have identified a functional NLS between amino acids 505 and 539. Sensitivity of wild-type ERK5 to leptomycin treatment, which is lost in the 409 mutant, further shows that the tail is required for a NES-dependent export mechanism. This result further indicates that wild-type ERK5 is continuously shuttling between nucleus and cytosol.

In a simplified model (Fig. 9), we can consider ERK5 to exist in closed and open conformations. Under steady-state conditions and in the absence of any factor, the majority of ERK5 is assumed to adopt the closed conformation. Under this condition, ERK5 activation by MEK5 and phosphorylation is hampered. Masking of the NLS and exposure of an NES (or co-export with an NES-containing protein) results in a dominant cytosolic localization. The open conformation, on the other hand, facilitates activation and is stabilized by activating phosphorylations. The additional exposure of the NLS overrides the weaker NES and leads to nuclear accumulation. Dephosphorylation of ERK5 by phosphatases triggers the return to the initial state. Deletion of the far C terminus (713 mutant) might be sufficient to destabilize the closed conformation, which could explain the observed hyperactivation and nuclear accumulation. In contrast to wild-type ERK5, the ERK2-ERK5 chimera seems to adopt an open rather than closed conformation, because addition of the tail did not affect kinase activity but induced an increase in nuclear accumulation, possibly by providing an additional import mechanism.

As expected, activated ERK5 can be found in the nuclei of breast cancer cells (22). However, Raviv *et al.* recently pointed out that, unlike ERK2, even non-activated ERK5 could be found in nuclei of rat fibroblasts (40). Nuclear import of ERK2 requires binding of the MEK1/2 containing an NLS (3, 41). In contrast, because it possesses its own NLS, ERK5 is self-competent to enter the nucleus. According to our hypothetical model, any factor capable of stabilizing the open conformation of ERK5 could thus trigger nuclear import of non-activated ERK5. It is noteworthy that a first activation-independent role for ERK5 was recently demonstrated. The intrinsic basal activity of ERK5 (but not necessarily its activation by MEK5) was shown to contribute to the survival of leukemia cells.²

ERK5 expression was found in virtually all kind of cells, indicating that ERK5, like other MAPKs, is most likely ubiquitously expressed. Rather than being restricted to a particular function in highly specialized cells, this might further indicate that ERK5 presumably serves in the regulation of diverse cellular functions depending on the cellular context. Indeed, in recent years, ERK5 was implicated in different cellular functions ranging from myoblast differentiation (14) to proliferation of breast cancer cells (25). Whether and to what extent the C-terminal tail of ERK5 contributes to its distinct functions remains to be addressed. The knowledge accumulated thus far about the ERK5 tail now allows the design of mutants that can be used to address very particular aspects of ERK5 function.

For instance, using the hyperactivated 713 mutant in combination with its functional or inactivated NLS will permit us to discriminate between cytosolic and nuclear functions of ERK5. Of extraordinary interest is the question of which mutants of ERK5 would be able to rescue the embryonically lethal phenotype of ERK5-deficient mice.

Acknowledgment—We would like to thank Dr. Silvio Gutkind for providing valuable reagents.

REFERENCES

- Manning, G., Plowman, G. D., Hunter, T., and Sudarsanam, S. (2002) *Trends Biochem. Sci.* **27**, 514–520
- Marshall, C. J. (1995) *Cell* **80**, 179–185
- Cobb, M. H., and Goldsmith, E. J. (2000) *Trends Biochem. Sci.* **25**, 7–9
- Whitmarsh, A. J., and Davis, R. J. (2000) *Cell. Mol. Life Sci.* **57**, 1172–1183
- Hazzalin, C. A., and Mahadevan, L. C. (2002) *Nat. Rev. Mol. Cell. Biol.* **3**, 30–40
- Sohn, S. J., Sarvis, B. K., Cado, D., and Winoto, A. (2002) *J. Biol. Chem.* **277**, 43344–43351
- Regan, C. P., Li, W., Boucher, D. M., Spatz, S., Su, M. S., and Kuida, K. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 9248–9253
- Selcher, J. C., Nekrasova, T., Paylor, R., Landreth, G. E., and Sweatt, J. D. (2001) *Learn. Mem.* **8**, 11–19
- Pages, G., Guerin, S., Grall, D., Bonino, F., Smith, A., Anjuere, F., Auburger, P., and Pouyssegur, J. (1999) *Science* **286**, 1374–1377
- Hayashi, M., Kim, S. W., Imanaka-Yoshida, K., Yoshida, T., Abel, E. D., Eliciri, B., Yang, Y., Ulevitch, R. J., and Lee, J. D. (2004) *J. Clin. Investig.* **113**, 1138–1148
- Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science* **241**, 42–52
- Lee, J. D., Ulevitch, R. J., and Han, J. (1995) *Biochem. Biophys. Res. Commun.* **213**, 715–724
- Zhou, G., Bao, Z. Q., and Dixon, J. E. (1995) *J. Biol. Chem.* **270**, 12665–12669
- Dinev, D., Jordan, B. W., Neufeld, B., Lee, J. D., Lindemann, D., Rapp, U. R., and Ludwig, S. (2001) *EMBO Rep.* **2**, 829–834
- Kato, Y., Chao, T. H., Hayashi, M., Tapping, R. I., and Lee, J. D. (2000) *Immunol. Res.* **21**, 233–237
- Watson, F. L., Heerssen, H. M., Bhattacharyya, A., Klesse, L., Lin, M. Z., and Segal, R. A. (2001) *Nat. Neurosci.* **4**, 981–988
- Kasler, H. G., Victoria, J., Duramad, O., and Winoto, A. (2000) *Mol. Cell. Biol.* **20**, 8382–8389
- Mody, N., Campbell, D. G., Morrice, N., Pegg, M., and Cohen, P. (2003) *Biochem. J.* **372**, 567–575
- Yan, C., Luo, H., Lee, J. D., Abe, J., and Berk, B. C. (2001) *J. Biol. Chem.* **276**, 10870–10878
- Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997) *EMBO J.* **16**, 7032–7044
- Buschbeck, M., Eickhoff, J., Sommer, M. N., and Ullrich, A. (2002) *J. Biol. Chem.* **277**, 29503–29509
- Esparis-Ogando, A., Diaz-Rodriguez, E., Montero, J. C., Yuste, L., Crespo, P., and Pandiella, A. (2002) *Mol. Cell. Biol.* **22**, 270–285
- Weldon, C. B., Scandurro, A. B., Rolfe, K. W., Clayton, J. L., Elliott, S., Butler, N. N., Melnik, L. I., Alam, J., McLachlan, J. A., Jaffe, B. M., Beckman, B. S., and Burow, M. E. (2002) *Surgery* **132**, 293–301
- Kato, Y., Kravchenko, V. V., Tapping, R. I., Han, J., Ulevitch, R. J., and Lee, J. D. (1997) *EMBO J.* **16**, 7054–7066
- Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J., and Lee, J. D. (1998) *Nature* **395**, 713–716
- Kamakura, S., Moriguchi, T., and Nishida, E. (1999) *J. Biol. Chem.* **274**, 26563–26571
- Abe, J., Takahashi, M., Ishida, M., Lee, J. D., and Berk, B. C. (1997) *J. Biol. Chem.* **272**, 20389–20394
- Cameron, S. J., Malik, S., Akaike, M., Lerner-Marmarosh, N., Yan, C., Lee, J. D., Abe, J., and Yang, J. (2003) *J. Biol. Chem.* **278**, 18682–18688
- Yang, S. H., Sharrocks, A. D., and Whitmarsh, A. J. (2003) *Gene* **320**, 3–21
- Mattaj, I. W., and Englmeier, L. (1998) *Annu. Rev. Biochem.* **67**, 265–306
- Nakielnny, S., and Dreyfuss, G. (1999) *Cell* **99**, 677–690
- Yashiroda, Y., and Yoshida, M. (2003) *Curr. Med. Chem.* **10**, 741–748
- English, J. M., Pearson, G., Baer, R., and Cobb, M. H. (1998) *J. Biol. Chem.* **273**, 3854–3860
- Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) *Nat. Cell Biol.* **2**, 110–116
- Bjorge, J. D., Jakymiw, A., and Fujita, D. J. (2000) *Oncogene* **19**, 5620–5635
- Smith, K. M., Yacobi, R., and Van Etten, R. A. (2003) *Mol. Cell* **12**, 27–37
- Pluk, H., Dorey, K., and Superti-Furga, G. (2002) *Cell* **108**, 247–259
- Carmo-Fonseca, M. (2002) *Cell* **108**, 513–521
- Xu, L., and Massague, J. (2004) *Nat. Rev. Mol. Cell. Biol.* **5**, 209–219
- Raviv, Z., Kalie, E., and Seger, R. (2004) *J. Cell Sci.* **117**, 1773–1784
- Pouyssegur, J., Volmat, V., and Lenormand, P. (2002) *Biochem. Pharmacol.* **64**, 755–763