MEK5 and ERK5 are localized in the nuclei of resting as well as stimulated cells, while MEKK2 translocates from the cytosol to the nucleus upon stimulation

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Summary

The ERK5 signaling cascade acts through sequential activation of MEKK2/3, MEK5 and ERK5 and transmits signals to a variety of stress and mitogenic related targets. In this study we examined the subcellular localization of the components of the ERK5 cascade and found that in resting, as well as in EGF-stimulated HeLa and Rat-1 cells, endogenous ERK5 is localized mainly in the nucleus. This location is different from the previously described location of exogenous ERK5, in the cytosol of resting cells, which is confirmed in this study. The reason for the different localization could be a saturation of anchoring moieties by the endogenous ERK5. Indeed, in situ detergent extraction analysis using Nonidet P-40, revealed that ERK5 is bound to detergent resistant moieties in the nucleus, while the exogenous protein fails to interact with those anchors. The upstream activator MEK5 is also localized in the nucleus both before and after EGF stimulation and is resistant to NP-40 extraction in resting cells. ERK5 remains bound to these nuclear moieties even after stimulation, while MEK5 is detached from the anchors but remains localized in the nucleus. Unlike ERK5 and MEK5, their upstream activator MEKK2 is localized mainly in the cytosol of resting cells, and translocates into the nucleus upon EGF stimulation, allowing transmission of signals to the nuclear MEK5. The nuclear localization of MEK5 and ERK5 is different from that of ERK1/2 and MEK1/2 in resting cells, indicating that each MAPK cascade uses distinct mechanisms to transmit extracellular signals to their nuclear targets.

Key words: MAP Kinase, ERK5, MEK5, MEKK2, Subcellular localization

Introduction

The mitogen-activated protein kinase (MAPK) signaling system plays an essential role in the transduction of extracellular signals to cytoplasmic and nuclear effectors that regulate various cellular processes (Chen et al., 2001; Johnson and Lapadat, 2002; Pouyssegur et al., 2002). This signaling system consists of several distinct protein kinase cascades including those that use extracellular signal-regulated kinase (ERK)1/2, Jun N-terminal kinase (JNK), p38MAPK and ERK5 for their action. The ERK5 (also known as big-MAPK; BMK1) signaling cascade acts through sequential activation of MAP3Ks (MEKK2/3 and Cot) (Chao et al., 1999; Chayama et al., 2001; Chiariello et al., 2000; Widmann et al., 1999), MEK5 and ERK5 (Lee et al., 1995; Zhou et al., 1995). Unlike the other MAPK cascades that seem to serve one set of extracellular stimuli such as stress or mitogens, the activation of the ERK5 cascade seems to be essential for both stress responses and proliferation (Abe et al., 1996; Kato et al., 1998). In addition, this cascade plays a role in the regulation of differentiation (Dinev et al., 2001), in neuronal survival (Watson et al., 2001), in embryonic angiogenesis (Sohn et al., 2002) and together with ERK1/2 in the induction of cancer (Pearson et al., 2001).

The ERK5 cascade seems to function mainly through regulation of transcription as do other MAPK signaling cascades. It has been shown that ERK5 directly phosphorylates and activates several transcription factors including c-Myc (English et al., 1998), MEF2 family members (Kato et al., 1997; Yang et al., 1998) and c-Fos (Kamakura et al., 1999). In addition, the transcription factor SAP1a, which mediates transcription from serum-response element, was shown to be activated in cells coexpressing ERK5 together with constitutively active MEK5 because of a direct phosphorylation by ERK5 (Kamakura et al., 1999). However, ERK5 was found to influence transcription not only by direct activatory phosphorylation, but also through additional mechanisms. One of these mechanisms seems to be the activation of protein kinases acting downstream of ERK5, as was shown for c-Fos (Terasawa et al., 2003) and NFkB (Pearson et al., 2001). Another way in which ERK5 influences transcription is through a kinase-independent mechanism that involves protein-protein interactions of its C-terminal non-catalytic half (Abe et al., 1996; Zhou et al., 1995). This important function of the C-terminal part of ERK5 was demonstrated with the transcriptional activation of MEF2C, which is enhanced by a direct association with the C-terminal part of ERK5 (Suzaki et al., 2002). Moreover, a recent study demonstrated that the ERK5 cascade causes stabilization of c-Fos and Fra-1 by mechanisms that include association with the C-terminal half of ERK5 (Terasawa et al., 2003). Finally, a unique feature of the ERK5 C terminus is its intrinsic transcriptional activity, which was shown to activate the Nur77

gene upon calcium signals in T cells (Kasler et al., 2000). This activity seems to be mediated by a constitutive recruitment of the ERK5 to MEF2 sites in the Nur77 promoter, and this singles out ERK5 in having two intrinsic catalytic activities rather than the one protein kinase activity of other MAPKs.

Although the ERK5 cascade may have cytosolic functions such as activation of SGK (Hayashi et al., 2001) and phosphorylation of connexin-43 (Cameron et al., 2003), most of the data to date indicate that its chief role relates to the regulation of transcription in the nucleus. Indeed, a study concerning the subcellular localization of ERK5 has demonstrated that GFP-ERK5 is localized in the cytoplasm of resting cells and translocates to the nucleus when coexpressed with a constitutively active form of MEK5 (Kato et al., 1997; Yan et al., 2001). However, in this study we examined in detail the subcellular localization of the endogenous ERK5 and found that in contrast to its exogenously expressed counterpart it is localized in the nucleus of HeLa and Rat-1 cells both before and after EGF stimulation. Similar localization was obtained when anti-MEK5 antibody was used for immunostaining. In situ detergent extraction using NP-40, which extracts non-anchored proteins, revealed that ERK5 and MEK5 are bound to NP-40 resistant moieties in the nucleus. MEKK2, the MAP3K kinase of the ERK5 cascade, was found mainly in the cytosol of resting cells and was shown to translocate into the nucleus upon stimulation. Thus, our results indicate that extracellular signals can be transmitted to the nuclear ERK5-MEK5 by MEKK2 that undergoes cytosol-nuclear shuttling upon mitogenic stimulation.

Materials and Methods

Materials and antibodies

Epidermal growth factor (EGF) and leptomycin B (LMB) were purchased from Sigma, Israel. Primary antibodies: rabbit anti-ERK5 and rabbit anti-MAPK (ERK-1, ERK-2) antibodies were obtained from Sigma. Goat anti-ERK5 (C-20) and (N-19), rabbit anti-ERK5 (H-300), goat anti-MEK5 (C-20), rabbit anti-MEKK2 (N-19), MEKK2 (C-18), rabbit anti-Sp1 (PEP2), mouse anti-Histone-1 (AE-4) and rabbit anti-caspase3 (H-277) antibodies were purchased from Santa Cruz (CA, USA). Rabbit anti-phospho-ERK5/BMK1 (pTpY218/220) antibody was purchased from BioSource (CA, USA). Rabbit anti-MEK5 antibody was purchased from Stressgen (CA, USA) and from Chemicon International (Temecula, CA). Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG, AP-conjugated goat anti-mouse IgG, peroxidase-conjugated goat anti-rabbit IgG, peroxidase-conjugated goat anti-mouse IgG, lissamine rhodamine (LRSC)-conjugated goat anti-rabbit IgG + IgM, LRSC-conjugated goat anti-mouse IgG + IgM and Cy3-conjugated donkey anti-goat IgG, were purchased from Jackson Immunoresearch (PA, USA)

Cell culture and transient transfection

HeLa and Rat-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 Units/ml penicillin and 50 μg/ml streptomycin. Sub-confluent cells were subjected to serum-starvation (0.1% FCS) for at least 18 hours and stimulated with epidermal growth factor (EGF) as indicated below. For transient transfection of HeLa cells we used the polyethyleneimine (PEI) method (Horbinski et al., 2001) for the expression vector pEGFP-C1 (CLONTECH Inc. UK) containing the cDNAs (inserted into the *Xho*I-*Hin*dIII sites) of wild-type (WT)-hERK5. pCMV5 plasmid containing rat MEK5α-1 with a 3×HA tag in its 3′ end (HA-WT-MEK5), Ser313Asp,Thr317Asp-MEK5α-1 (HA-MEK5D a constitutively active MEK5), in the 3×HA containing plasmid (Kato et al., 1997) and Ser313Ala,Thr317Ala-MEK5α-1 in the same plasmid (HA-AA-MEK5, inactive) were a generous gift from Dr J. D. Lee (Dept. of Immunology, The Scripps Institute, La Jolla, CA).

Immunostaining

HeLa or Rat-1 cells were grown on glass coverslips under the conditions described above. After serum-starvation the cells were treated with various reagents and then washed twice with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde (PFA) for 20 minutes, and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes. For MEKK2 staining in Rat-1 cells, the cells were fixed with methanol and acetone (1:1) for 10 minutes at –20°C. Next, the cells were incubated with the relevant primary antibodies for 45 minutes. For competition analysis, blocking peptide (200 μg/ml) was incubated together with the relevant primary antibody. This was followed by three washes with PBS and incubation with a rhodamine-conjugated secondary antibody for an additional 45 minutes together with 4′, 6′-diamidino-2-phenylindole (DAPI) for DNA staining. Then, the coverslips were washed again three times with PBS and were mounted for examination. For GFP studies, fixation and permeabilization of the cells were performed as described above but the cells were stained with DAPI without any additional antibody. The fluorescence imaging was performed using a Nikon (EFD-3) fluorescence microscope at ×400 magnification.

Detection of protein kinase activation

Serum-starved cells were treated as indicated, washed twice with PBS and once with buffer A (50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM sodium vanadate). Cells were lysed in RIPA buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) for 5 minutes on ice then scraped into Eppendorf tubes. Cell lysates were centrifuged at 20,000 ***g*** for 15 minutes at 4°C. Then, a sample buffer was added followed by boiling, and similar amount of proteins from each fraction were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 2% BSA (Sigma) for 30 minutes. The blots were developed with either alkaline phosphatase (AP) or horseradish peroxidase (HRP).

Nuclear and cytosolic fractionation

The preparation of nuclear and cytosolic fractions was modified from the procedure described by Meunier et al. (Meunier et al., 2002). Briefly, HeLa and Rat-1 cells were grown in 10 cm plates to reach sub-confluency, serum-starved (0.1%, 18 hours) and either activated with EGF or left untreated as control. Then the cells were washed with cold PBS, suspended in ice-cold buffer F (210 mM mannitol, 70 mM sucrose 5 mM Tris, pH 7.5, 1 mM EDTA, supplemented with protease inhibitors) left in the buffer for 15 minutes on ice and then dounce homogenized (15 strokes). The nuclei were separated by centrifugation (500 ***g***, 5 minutes, 4°C). The supernatant, containing the cytosolic fraction, was boiled in sample buffer. The pellet, containing the nuclei, was washed with PBS and then resuspended in RIPA buffer for 5 minutes on ice, centrifuged again and the supernatant (nuclear fraction), was boiled in sample buffer. The same volumes of nuclear or cytosolic fractions were analyzed.

In situ NP-40 extraction

The procedure was carried out as described by Andegeko et al. (Andegeko et al., 2001), with minor changes. Briefly, Rat-1 cells were grown on coverslips to reach sub-confluency. Cell extraction was

carried out in situ by incubating the coverslips in fractionation buffer (FB) containing 50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 (NP-40), 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and protease inhibitors for 20 minutes on ice. The buffer was removed, and the procedure was repeated twice more on ice for 10 minutes and 5 minutes. For HeLa cells, FB containing 0.2% NP-40 was added and the cells were incubated on ice for a maximum of 5 minutes. Both types of cells were then fixed, permeabilized and stained as describe above.

Results

ERK5 is localized in the nucleus of resting as well as stimulated Rat-1 and HeLa cells

The subcelular localization of components of the MAPK cascades is known to play an important role in the regulation of the signals transmitted by these cascades (Pouyssegur et al., 2002). We initiated cell-staining experiments to study the localization of ERK5. Thus, staining of serum-starved Rat-1 cells with anti-ERK5 antibody directed to the C terminus of ERK5 visualized a nuclear distribution with very little fluorescence in the cytoplasm of these cells. This staining was specific because competition with the antigenic peptide of the antibody completely abolished this nuclear staining (Fig. 1A). Since other MAPKs (Seger and Krebs, 1995) as well as overexpressed ERK5 (Yan et al., 2001) seem to be localized primarily in the cytoplasm of resting cells, it was important to validate this nuclear localization of the endogenous ERK5. To this end, we stained Rat-1 cells with additional antibodies against different epitopes of ERK5. Indeed, staining with antibody to the N terminus (N-19), C terminus from another company (C-20) and to a central region of the molecule (H-300) all resulted in a nuclear staining with only a trace staining in other compartments (Fig. 1B). Similar to Rat-1 cells, major nuclear staining with anti-ERK5 antibody was observed also in EJ, COS7 (data not shown) and HeLa cells (Fig. 2A,B, NS). The nuclear staining was detected in spite of the low amount of activated ERK5 molecules in all cells (Fig. 2C,D, NS and data not shown).

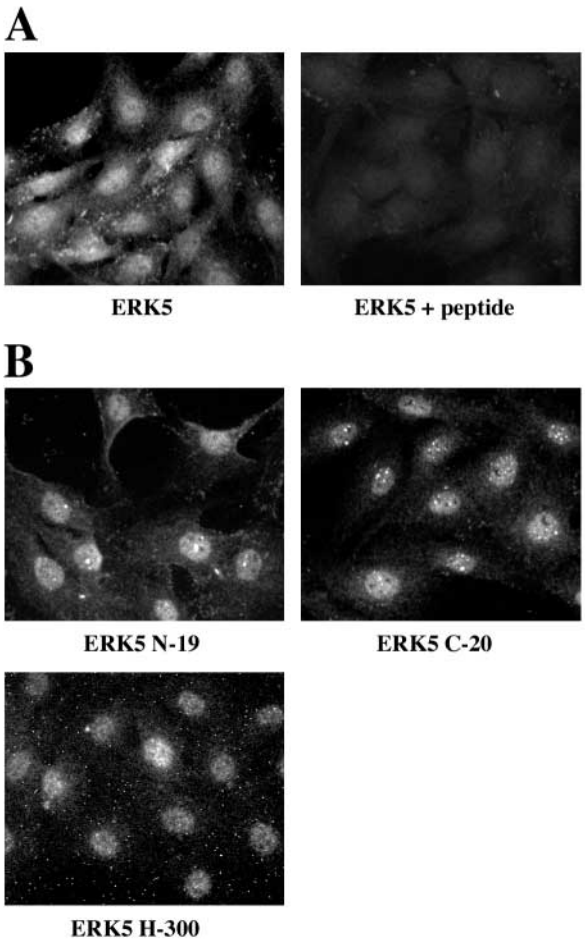
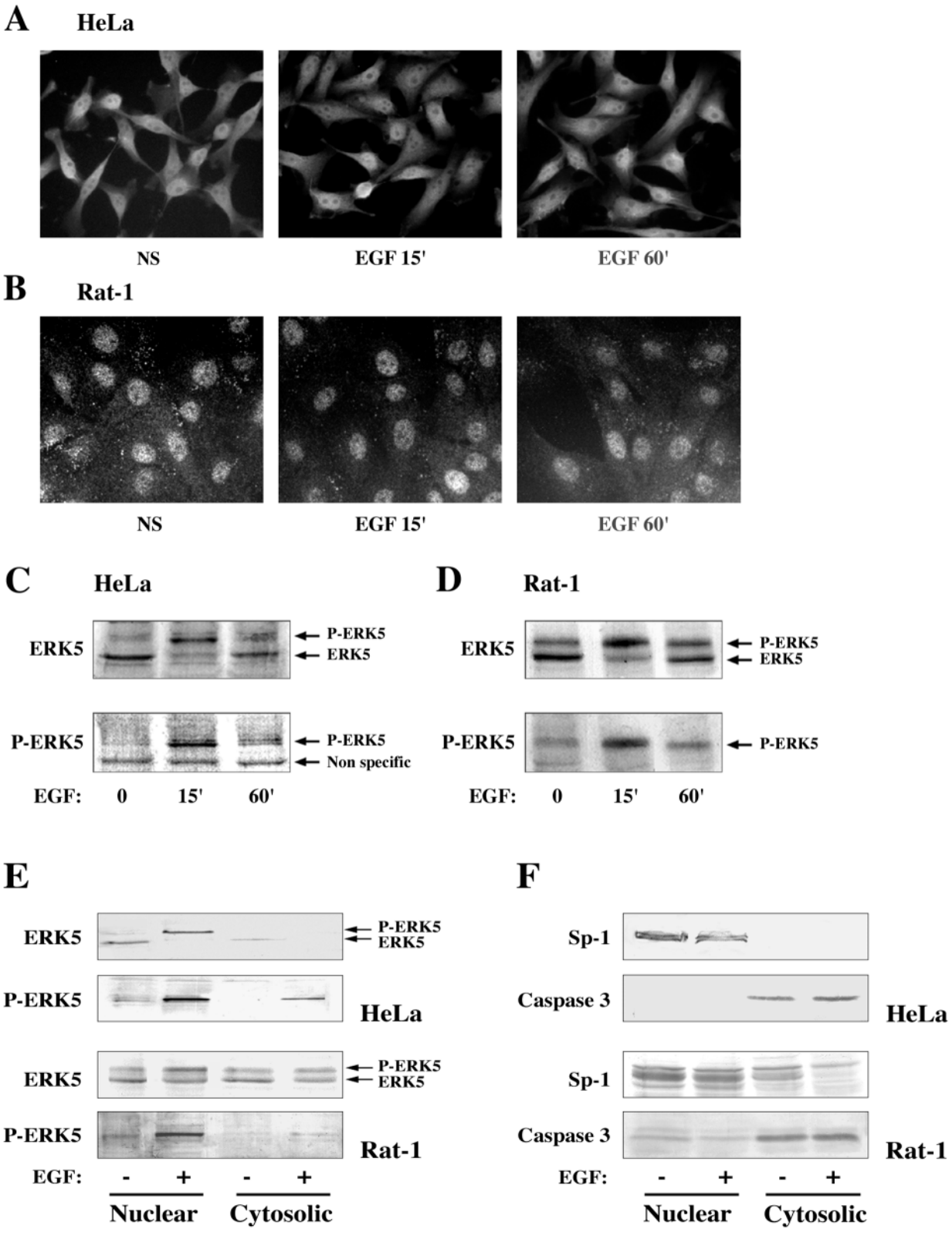


Fig. 1. ERK5 is localized in the nucleus in Rat-1 cells. (A) Rat-1 cells were grown on coverslips, fixed with PFA, permeabilized with 0.2% Triton X-100 and stained with rabbit anti-ERK5 antibody directed to the C terminus of ERK5 in the presence or absence of blocking peptide (50 μg/ml). (B) Rat-1 cells were treated as described above and stained with antibodies to different epitopes of ERK5 (N-19, C-20, H-300).

Since MAPKs are known to change their distribution upon stimulation (Pouyssegur et al., 2002; Seger and Krebs, 1995), it was important to examine the effect of EGF, a stimulator of ERK5 activity (Kato et al., 1998), on its localization. Our results (Fig. 2) demonstrate that EGF treatment did not induce any significant change in the subcellular distribution of ERK5 up to 60 minutes of stimulation, in both HeLa (Fig. 2A) and Rat-1 (Fig. 2B) cells. This lack of effect on the subcellular localization occurred in spite of the clear activation of ERK5 upon EGF stimulation in both cell lines (Fig. 2C,D). To further confirm the nuclear staining of ERK5 we used a cellular fractionation approach, which was shown to be efficient in separating the nuclear marker Sp-1 from the cytosolic marker caspase 3 in both HeLa and Rat-1 cells (Fig. 2E,F). As expected from the previous results, the fractionation revealed nuclear localization of both inactive and stimulated ERK5, although some ERK5 as well as Sp-1 was detected also in the cytoplasm of Rat-1 cells probably due to leakage during preparation.

Exogenous GFP-ERK5 is localized in the cytosol of HeLa and Rat1 cells

In contrast to the nuclear localization of ERK5 observed in Rat-1 and HeLa cells, previous studies have demonstrated that overexpression of GFP-ERK5 results in its cytoplasmic localization, which can turn into nuclear distribution upon coexpression with the constitutively active form of MEK5 (MEK5D) (Kato et al., 1997; Yan et al., 2001). We repeated these experiments in HeLa cells, and indeed found that when GFP-ERK5 was expressed, it was detected mainly in the cytosol (Fig. 3A). Coexpression with the constitutively active MEK5D induced translocation of GFP-ERK5 into the nucleus, without a concomitant change in distribution of the mutant MEK5 (data not shown). However, coexpression of WT-MEK5 or AA-MEK5 as well as stimulation with EGF had no effect on the cellular distribution of overexpressed ERK5 (Fig. 3A). This distribution of GFP-ERK5 was unrelated to the GFP portion of the GFP-ERK5 since transiently overexpressed HA-ERK5 showed similar cytoplasmic staining and GFP alone was diffusely distributed all over the cells (data not shown). The translocation of the GFP-ERK5 into the nucleus was correlated with enhanced phosphorylation of the regulatory tyrosine and threonine as well as other residues, as judged from the

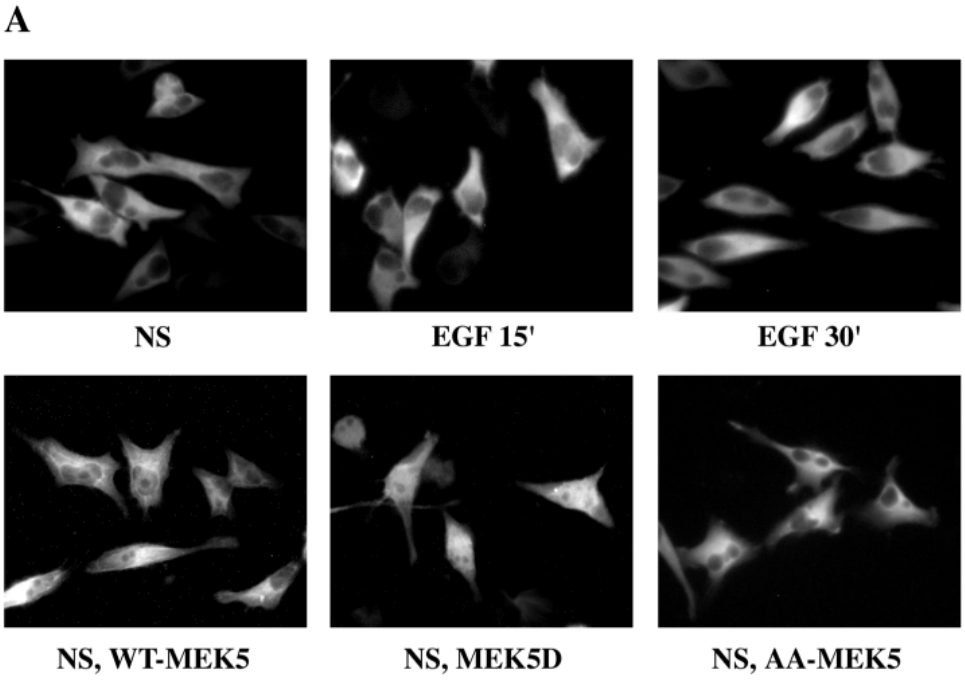


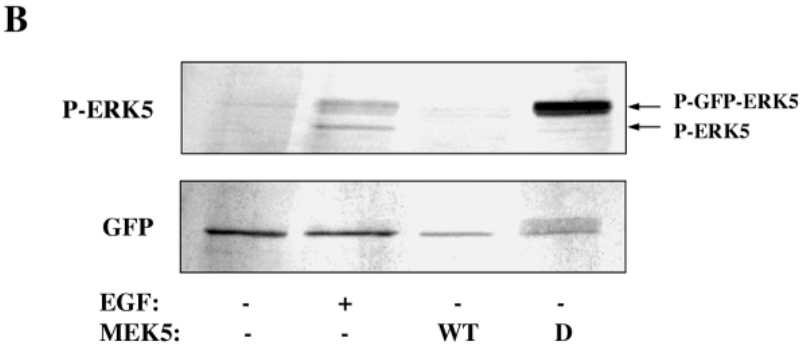
increased staining by the anti-phospho-ERK5 antibodies and the upshift of GFP-ERK5 obtained in the cells cotransfected with MEK5D (Fig. 3B). Therefore, it is possible that the translocation could be an outcome of several distinct phosphorylations on ERK5, which were recently reported by Mody et al. (Mody et al., 2003) to occur upon cellular stimulation.

The different localization of exogenous and endogenous ERK5 resembles in part the different localization of exogenous and endogenous ERK1/2. It is well-demonstrated that

Fig. 2. ERK5 is localized in the nucleus before and after stimulation. HeLa cells (A) or Rat-1 cells (B) were grown on coverslips, serum-starved and activated with 50 ng/ml EGF for the indicated times. The treated cells were then fixed and stained with anti-ERK5 antibody (N-19). Then the cells were tested for ERK5 phosphorylation. For this purpose, HeLa cells (C) or Rat-1 cells (D) were grown in 6 cm tissue culture plates and activated with 10 ng/ml EGF for the indicated times. After treatment, the cells were harvested with RIPA buffer, and cell lysates were separated by SDS-PAGE and subjected to western blots analysis with anti-ERK5 or anti-phospho-ERK5 (P-ERK5) antibodies. Biochemical fractionation of ERK5 was performed as described under Materials and Methods. (E) HeLa cells (upper panels) or Rat-1 cells (lower panels), were grown in 10 cm tissue culture plates, serum-starved and activated with EGF (50 ng/ml, 15 minutes). Then the cells were washed with ice cold PBS and fractionated into nuclear and cytoplasmic fractions. Equal volume aliquots of each fraction were subjected to SDS-PAGE and western blotting and analyzed with anti-ERK5 (C terminus) and anti-phospho-ERK5 (P-ERK5) antibodies. (F) The samples described in E were subjected to western blot analysis with anti-Sp-1 and anti-caspase 3 antibodies.

endogenous ERK1/2 are localized in the cytosol of resting cells, while exogenous ERK1/2 are detected solely in the nucleus (Fukuda et al., 1997b; Rubinfeld et al., 1999). The differential localization of ERK1/2 is probably related to a limited amount of anchoring proteins (including MEK1/2) in the cytosol, which do not allow binding of all exogenously expressed ERK1/2 that are then translocated into the nucleus (Wolf et al., 2001). Indeed, when we stably expressed HA-ERK5 in Rat1 and HeLa cells and studied the subcellular localization with anti-HA antibodies we found that the HA-ERK5, which was expressed in lower amounts than the transiently expressed GFP-ERK5, was distributed all over the cells, with some preference to the nucleus (data not shown). These results are consistent with the notion that endogenous ERK5 saturates most of the nuclear anchoring moieties that are responsible for the nuclear localization of this protein. A limited number of unoccupied anchoring moieties are able to bind only a small portion of the highly overexpressed ERK5 (transient transfection) and a bigger portion of the moderately overexpressed HA-ERK5 in the stable transfection, resulting in their distinct distributions.





ERK5 is retained in the nucleus by NP-40-resistant anchoring moieties

To further address the possibility that ERK5 is retained in the nucleus by anchoring moieties, we incubated HeLa cells with NP-40 for an 'in situ detergent extraction', under conditions where unbound cytosolic and nuclear proteins are extracted, but cytoskeletal and bound proteins remain intact on the slide (Andegeko et al., 2001). As expected, ERK5 was localized mainly in the nuclei of HeLa cells,

Fig. 3. Exogenous GFP-ERK5 is localized in the cytoplasm. (A) HeLa cells were grown on coverslips, transfected with GFP-ERK5 construct (2.5 μg) or GFP-ERK5 (2.5 μg) together with either HA-WT-MEK5, HA-AA-MEK5 or HA-MEK5D (2.5 μg each) using the PEI method (Materials and Methods) and left untreated for additional 24 hours. This was followed by serum-starvation (0.1%) for 18 hours, after which the cells were left either unstimulated (NS) or stimulated with EGF (50 ng/ml, 30 minutes). Then, the cells were washed with PBS, fixed with PFA, and the GFP was visualized using a fluorescence microscope. (B) HeLa cells were grown in 6 cm tissue culture plates and transfected with GFP-ERK5 alone (4 μg) and together with either WT-MEK5 (WT, 4 μg) or HA-MEK5D (D, 4 μg). As above, the cells were left to recover, serum-starved, and either stimulated with EGF (50 ng/ml, 30 minutes) or left unstimulated (NS). Then the cells were harvested and subjected to western blotting with anti P-ERK5 and GFP antibodies.

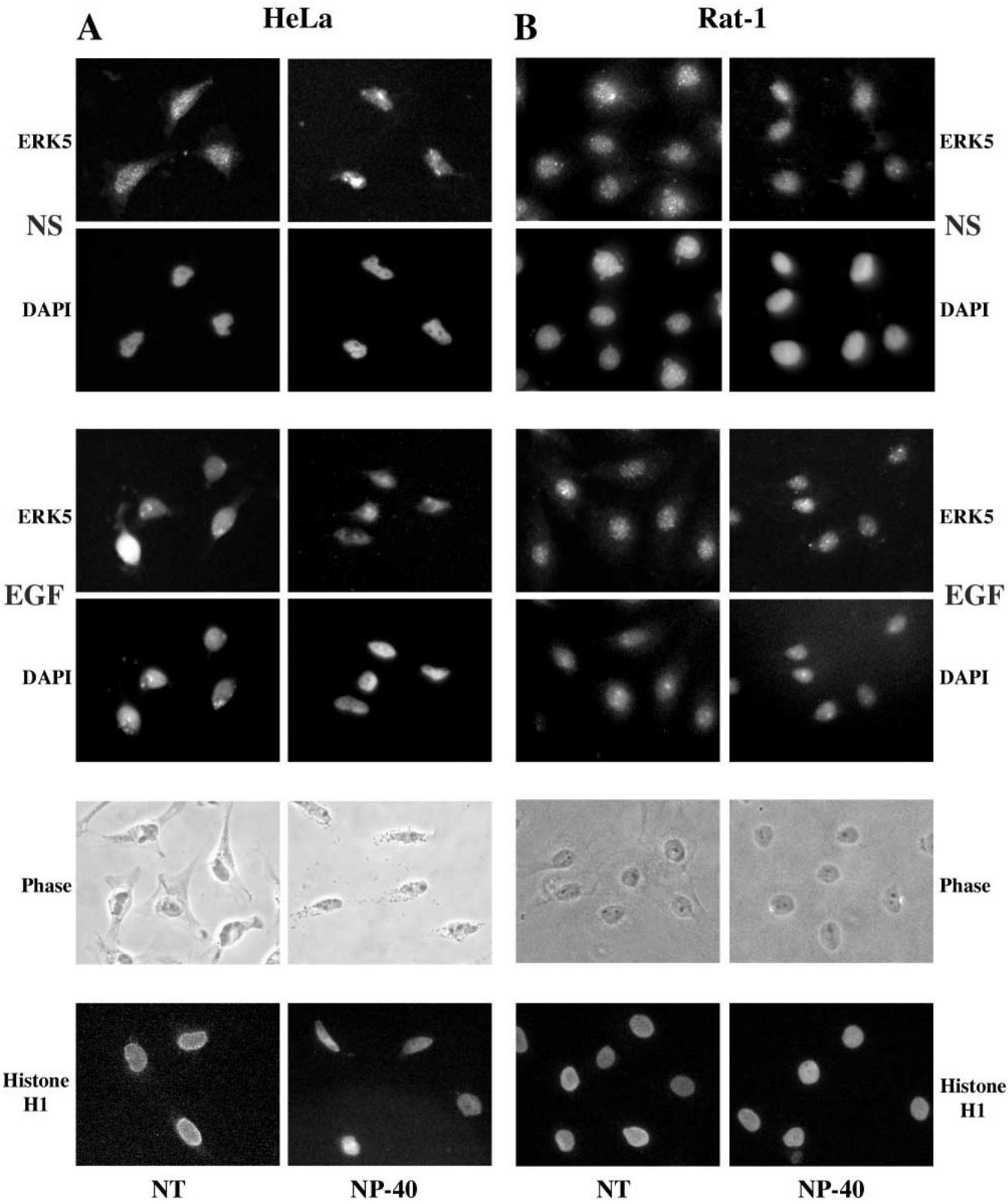


Fig. 4. Endogenous ERK5 is retained in the nucleus after in situ detergent extraction. (A) HeLa cells were grown on coverslips as described before, serum-starved and stimulated with EGF (50 ng/ml, 15 minutes) or left untreated. The cells were then subjected to an in situ detergent extraction with NP-40 (Materials and Methods, 0.2%, 5 minutes). The fixed cells were stained with goat anti-ERK5 antibody (N19) and with DAPI. Phase contrast micrographs of the non-activated NP-40 untreated and treated cells demonstrates the effect of the detergent extraction. Staining with anti-histone H1 antibodies is shown as a positive control in the lower panel. (B) Rat-1 cells were grown and treated as described in A, except that the in situ NP-40 extraction used 0.5% for 10 minutes.

and this nuclear location was kept even after the NP-40 treatment (Fig. 4A). This did not changed upon EGF stimulation and occurred despite the massive NP-40 extraction of cellular components, as detected by phase contrast analysis (Fig. 4A). Staining for histone H1 served as a positive control for the method, and showed that nuclear retention can indeed be detected upon the in situ NP-40 extraction (Fig. 4A). Similar results were obtained also in Rat-1 cells, where both ERK5 and histone H1 were retained in the nucleus (Fig. 4B) in an NP-40 concentration that was optimized for Rat-1, which was higher than the optimum concentrations for HeLa cells (0.5% vs 0.2%, respectively).

Since the overexpressed GFP-ERK5 demonstrated a different localization than that of the endogenous ERK5, it was important to identify whether the exogenous protein also interacts with any anchoring proteins in either the cytosol or the nucleus. We therefore first incubated GFP-ERK5-transfected HeLa cells with LMB, which prevents export from the nucleus. As shown in Fig. 5A, in these cells the GFP-ERK5 accumulated in the nucleus, indicating that GFP-ERK5 may undergo cyto-nuclear shuttle without any cellular stimulation. In addition, when we applied the in situ NP-40 extraction method to the GFP-ERK5-transfected HeLa cells, the GFP-ERK5 staining was abolished in the LMB-treated as well as in untreated cells (Fig. 5B). These results indicate that GFP-ERK5 is not strongly bound to any NP-40-resistant anchoring proteins in the cells, and although GFP-ERK5 can translocate into the nucleus, it is not retained there by the putative anchors, probably because the endogenous ERK5 is already bound to them. It should be noted that the in situ NP-40 extraction serves in fact as a positive control for the endogenous ERK5, and our results clearly indicate that proteins that are not strongly bound can be easily extracted from the nucleus under the conditions used. Taken together, these results strongly indicate that endogenous ERK5 is indeed retained in the nucleus by certain nuclear moieties, and this is the reason for its persistent nuclear localization before and after stimulation.

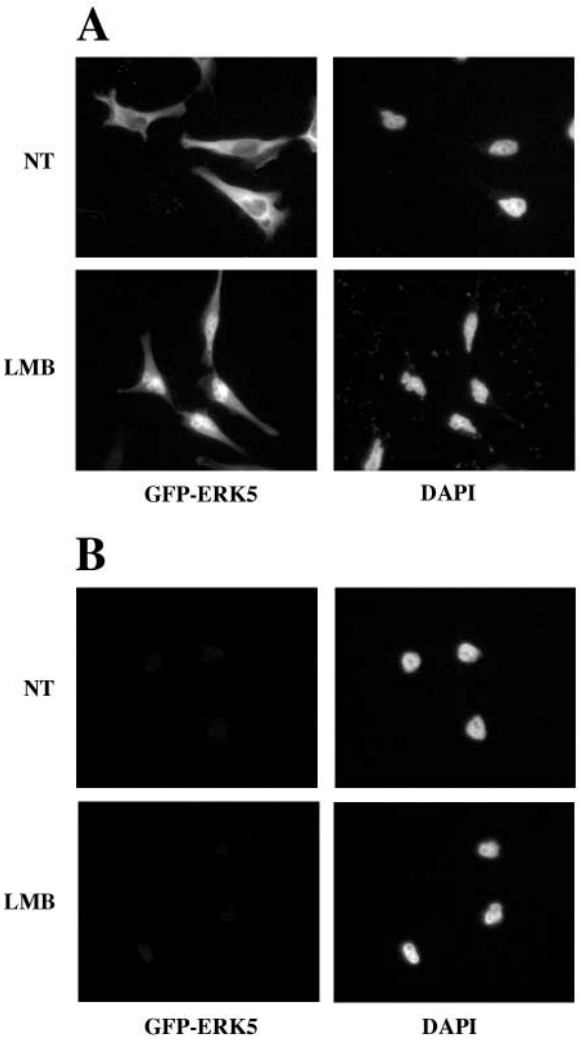


Fig. 5. GFP-ERK5 accumulates in the nucleus without anchoring upon LMB treatment. (A) HeLa cells were grown on coverslips and transfected with GFP-ERK5 construct (2.5 μg DNA) using the PEI method. The cells were either treated with LMB (5 ng/ml, 1 hour) or left untreated (NT), and then were washed with PBS, fixed with 3% PFA, permeabilized with 0.2% Triton X-100 and stained with DAPI. (B) HeLa cells were grown and treated as in A. After LMB treatment, cells were subjected to an in situ detergent extraction with NP-40 (0.2%, 5 minutes), stained with DAPI and examined with a fluorescence microscope.

Persistent localization of MEK5 in the nucleus of HeLa and Rat-1 cells

Similarly to ERK5, the subcellular localization of MEK5 was examined using specific antibodies. Interestingly, unlike other MAPKKs (Pouyssegur et al., 2002), MEK5 was detected in the nucleus of HeLa (Fig. 6A) as well as Rat-1 (Fig. 6B) cells, both before and after stimulation with EGF. Unlike, the staining of ERK5 that usually detected small amount of this kinase in some cytoplasmic compartments as well, the staining of MEK5 appears to be confined to the nucleus with a minor accumulation in unidentified nuclear spots. There was no significant detection in other cellular compartments. The nuclear staining appears to be specific, because it was competed out by the antigenic peptide (Fig. 6B), the antibody recognizes only MEK5 in a western blot (Fig. 6C) and because it was reproduced with other anti-MEK5 antibodies (Chemicon, Santa Cruz, data not shown).

We then repeated the in situ NP-40 extraction experiments for MEK5. In both HeLa and Rat-1 cells the staining with anti-MEK5 antibody after the NP-40 extraction resulted in nuclear localization with some accumulation in the spots seen also in the untreated cells. Interestingly, in both cell lines MEK5 was not retained in the nucleus of EGF-stimulated cells after the NP-40 extraction (Fig. 7A,B). These results may indicate that MEK5 is localized in the nuclei of resting cells bound to some NP-40-resistant anchors, that, because of the strong interaction with ERK5 (Zhou et al., 1995), might be the nuclear ERK5 molecules themselves. However, after EGF stimulation, MEK5 is detached from its anchors and participate in the activation of ERK5 in the nucleus.

Cytosolic MEKK2 translocates into the nucleus of HeLa and Rat-1 cells upon EGF stimulation

Since our results demonstrated that both MEK5 and ERK5 are

localized in the nuclei of HeLa and Rat-1 cells, it was important to identify the mechanism that allows the transduction of extracellular signals to the nuclear molecules. One possible mechanism could be a shuttle of MEK5 or another nuclear component to the cytosol where it receives the signal and then translocates back to the nucleus. To eliminate this possibility, we used LMB and examined the time course of ERK5 activation by EGF. Interestingly, the activation phase of ERK5 (Fig. 8, 0-25 minutes) was not affected by LMB treatment, while the inactivation phase was clearly inhibited (Fig. 8, 30-45 minutes), causing a prolonged ERK5 signal. This result indicates that the signal cannot reach the nucleus by a shuttle of a nuclear protein. Therefore, it is likely that the signal reaches the nucleus via translocation of an upstream activator of MEK5. The slower dephosphorylation of ERK5 in the nucleus of LMB pre-treated cells (Fig. 8) can be explained by a longer nuclear accumulation of the upstream shuttling compound that normally is exported out of the nucleus in the inactivation phase of the ERK5 cascade.

To further address the question of how the ERK5 cascade signal is transmitted to the nuclear MEK5-ERK5, we examined the subcellular localization of MEKK2 that together with MEKK3 has recently been implicated as the MAP3K of the cascade (Chao et al., 1999; Chayama et al., 2001; Sun et al., 2003). Using appropriate antibodies, we found MEKK2 all over the cells, indicating that MEKK2 is equally localized in the

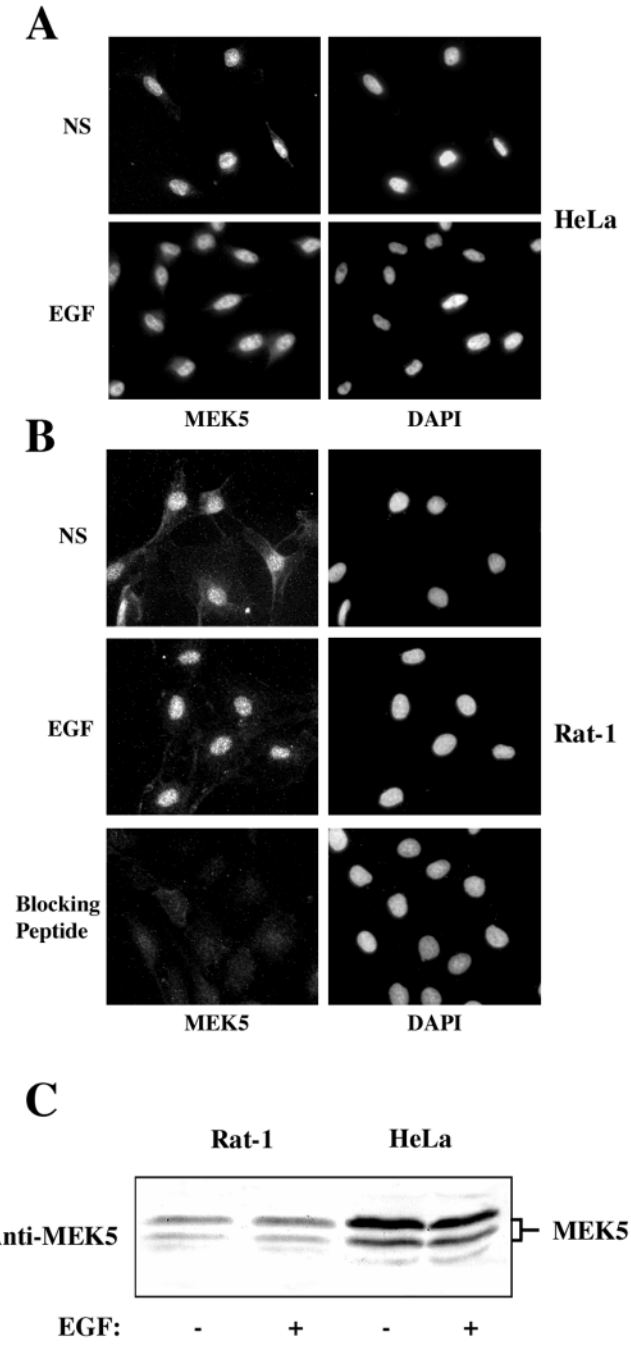


Fig. 6. MEK5 is localized in the nucleus. (A) HeLa cells were grown as described above, serum-starved, and then either stimulated with EGF (50 ng/ml, 15 minutes) or left unstimulated (NS). Then the cells were washed, fixed and stained with anti-MEK5 antibody and DAPI. (B) Rat-1 cells were grown and stimulated as described above, and then stained with anti-MEK5 antibody and DAPI. A blocking peptide (50 μg/ml) was added to the staining (lower panel) for competition. (C) Specificity of the anti-MEK5 antibody was demonstrated by western blot with extracts (30 μg) from HeLa and Rat-1 cells.

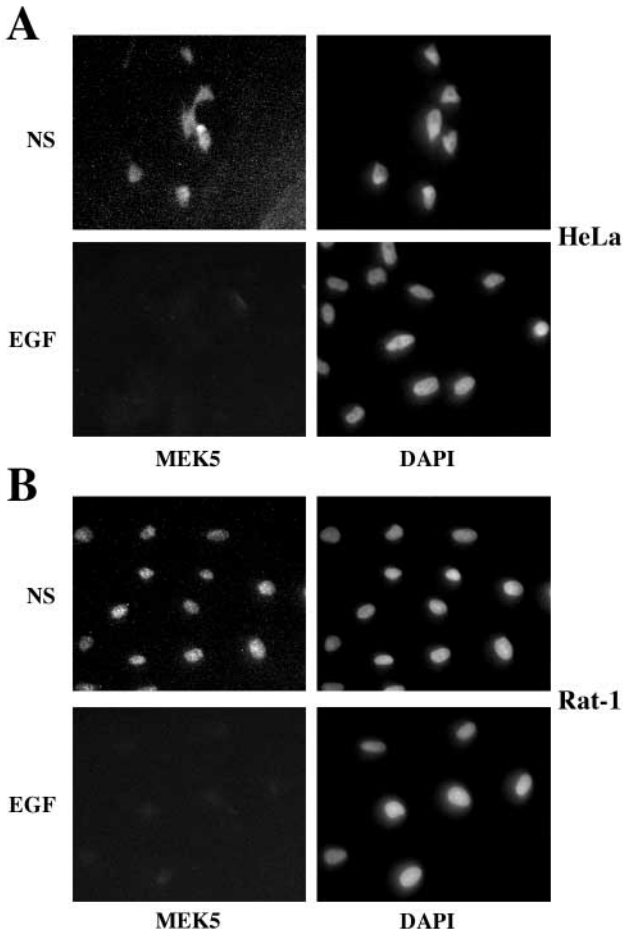


Fig. 7. MEK5 is retained in the nucleus of resting but not of stimulated cells. HeLa (A) or Rat-1 (B) cells were grown on coverslips as described above, serum-starved and either stimulated with EGF (50 ng/ml, 15 minutes) or left unstimulated (NS). Then the cells were subjected to an in situ extraction with NP-40 (0.2% for HeLa and 0.5% for Rat-1, 5 minutes), after which the cells were fixed and stained with anti-MEK5 antibody and DAPI.

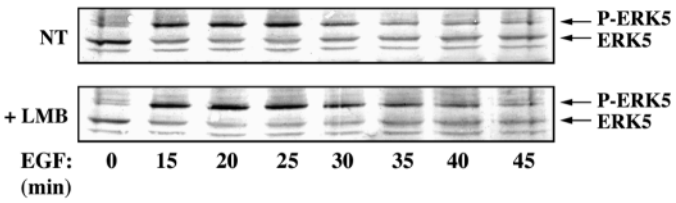


Fig. 8. LMB does not affect ERK5 activation but does affect its down regulation. HeLa cells were grown in 6 cm plates, serum-starved and preincubated with LMB (5 ng/ml, 1 hour) or left untreated (NT), followed by EGF stimulation (50 ng/ml) for the indicated times. Then, the cells were harvested in RIPA buffer and the lysates were separated by SDS-PAGE and subjected to a western blot analysis with anti-ERK5 antibody (N-19).

cytosol and in the nucleus of these cells (Fig. 9A). The staining was specific because it was competed by the antigenic peptide and because the antibodies recognized only MEKK2 in a western blot analysis (data not shown). We then examined the localization of MEKK2 upon EGF stimulation and found that 15 minutes after the addition of EGF, MEKK2 was primarily localized in the nuclei of the treated cells. This distribution changed back to the initial all-over pattern 60 minutes after EGF stimulation (Fig. 9A) indicating that MEKK2 can shuttle between the cytosol and the nucleus upon EGF stimulation.

Similar results were obtained when the subcellular distribution of MEKK2 was examined in Rat-1 cells (Fig. 9A lower panels). Interestingly, under different staining conditions, MEKK2 was also detected in some cytosolic organelles (possibly adhesion plaques, data not shown), but the nature and function of this localization are not yet clear. EGF stimulation induced nuclear accumulation of the MEKK2 within 15 minutes, and after 60 minutes the staining was again similar to the initial staining of the non-stimulated cells. As would be expected from the cyto-nuclear shuttling, when LMB was added to resting HeLa or Rat-1 cells, MEKK2 appeared mainly in the nucleus (Fig. 9B). Addition of LMB to the cells for 15 minutes followed by EGF stimulation, did not change the nuclear localization of MEKK2 as compared to the localization observed with EGF alone or with LMB alone. However, LMB did prevent the nuclear export of MEKK2 1 hour after EGF stimulation. Therefore, these results support the notion that MEKK2 is the enzyme that transmits extracellular signals to the nuclear MEK5 and ERK5, and that this enzyme is exported from the nucleus at later stages of mitogenic stimulation, thus participating in the inactivation of the ERK5 cascade.

Discussion

The proper subcellular localization of signaling kinases is important for the accurate and rapid transmission of intracellular signals through MAPK cascades. The best studied localization of signaling molecules is that of components of the ERK1/2 cascade (Pouyssegur et al., 2002; Seger and Krebs, 1995). Studies on these components demonstrated a cytosolic localization of Raf1, MEK1/2 and ERK1/2. However, stimulation of the cells results in a rapid translocation of Raf1 to the plasma membrane and of MEK1/2 and ERK1/2 into the nucleus. While ERK1/2 are retained in the nucleus by anchoring proteins (Lenormand et al., 1998), MEK1/2 are rapidly exported from that location because of their nuclear export signal (Fukuda et al., 1997a; Jaaro et al., 1997). By analogy to the ERK1/2 cascade, it was initially speculated that ERK5 is also retained in the cytosol of resting cells and translocates into the nucleus upon stimulation. This assumption was supported by the fact that overexpressed ERK5 was localized in the cytosol of resting cells, and shifted to a nuclear localization when coexpressed with a constitutively active MEK5 (Kato et al., 1997; Yan et al., 2001). In addition, it was also shown that in MCF7, BT474 and SKBR3 cells, active ERK5 is localized in the nucleus, although the distribution of inactive ERK5 was not determined (Esparis-Ogando et al., 2002).

In the current study we show that in fact, not only the active ERK5, but also the inactive ERK5 molecules are localized in the nucleus of resting Rat-1 and HeLa cells. We show that in these cells, ERK5 is localized in the nucleus even when most of its molecules are not phosphorylated on the regulatory threonine and tyrosine in the TEY motif and thereby are not activated. The nuclear localization of the inactive ERK5 was confirmed here by several methods, including (i) staining with four distinct antibodies (Figs 1, 2), (ii) competing the staining with the antigenic peptide (Fig. 1), and (iii) biochemical fractionation (Fig. 2E,F). All these methods show that non-stimulated, endogenous ERK5 is localized in the nucleus of the examined cells. These results are in line with the presence of nuclear localization sequence within ERK5, which in most proteins results in a constant nuclear localization (Gorlich, 1997). In addition, similar distribution of inactive ERK5 was detected by us in EJ and COS7 cells (data not shown), and in a previous study (Esparis-Ogando et al., 2002), the nuclear localization of ERK5 in BT474 cells was observed in spite of high amount of inactive ERK5 presence in those cells. Finally, the nuclear localization of ERK5 is supported also by (i) the fact that ERK5 has mainly nuclear functions (see Introduction), (ii) the ability of the C terminus of ERK5 to interact with nuclear moieties (Kasler et al., 2000), and (iii) the localization of the upstream activator (MEK5) in the nucleus (Fig. 6). The fact that ERK5 is localized in the nucleus both before and after stimulation (Fig. 2) and that under both conditions it is resistant to in situ detergent extraction (Fig. 4) indicates that ERK5 is strongly bound to anchoring moieties in the nucleus independent of its activation state.

In contrast to the nuclear localization of endogenous ERK5, the exogenous ERK5 appears to be localized in the cytosol of resting cells. This localization was previously observed in several cell types (Esparis-Ogando et al., 2002; Kato et al., 1997; Yan et al., 2001) and was reproduced here in HeLa cells (Fig. 3). Interestingly, stimulation with EGF was not sufficient to induce any translocation of the exogenous protein, and only coexpression with MEK5D, resulted in a diffused distribution both in the cytosol and the nuclei of HeLa cells. This result may suggest that just phosphorylation of the regulatory threonine and tyrosine is not sufficient to cause nuclear translocation of ERK5, and it is possible that additional phosphorylation events (Mody et al., 2003), or expression of anchoring proteins (Buschbeck et al., 2002) play a role in the partial translocation observed.

The cytosolic localization observed with the exogenous ERK5 raises the question as to what is the reason for the differences in the subcellular localization between the

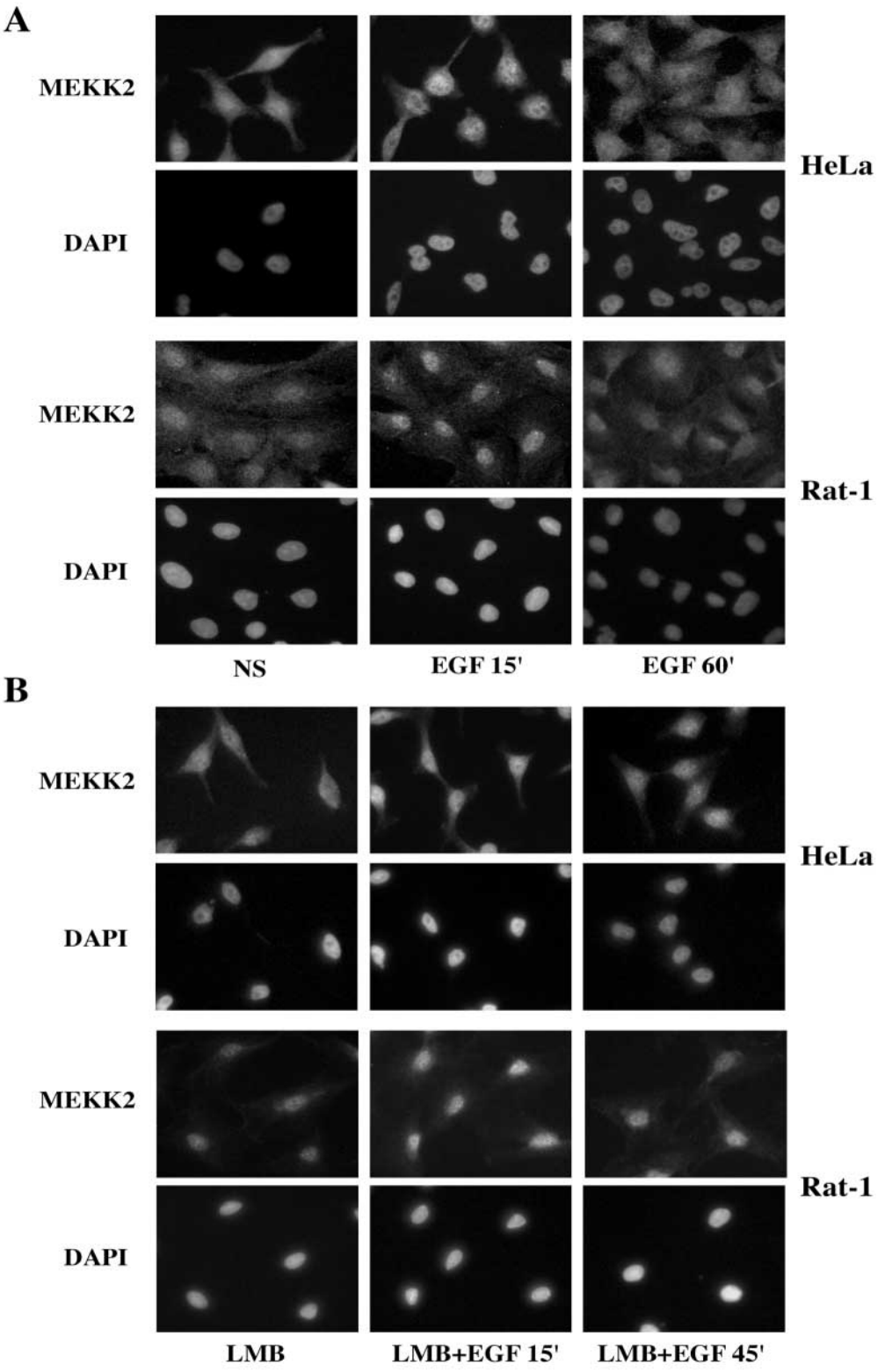


Fig. 9. MEKK2 translocates to the nucleus upon EGF stimulation. A. HeLa and Rat-1 cells were grown on coverslips, serum-starved and either stimulated with EGF (50 ng/ml) for the indicated times or left unstimulated (NS). Then the cells were washed, fixed and stained with anti-MEKK2 antibody (N-19) and with DAPI. (B) HeLa and Rat-1 cells were grown as for A, and incubated with LMB (5 ng/ml, 1 hour) followed by stimulation (EGF, 50 ng/ml) for the indicated times or left without FGF stimulation. Then the cells were washed, fixed and stained with anti-MEKK2 antibody and with DAPI.

endogenous and exogenous ERK5. A clue came from the localization of exogenous ERK1/2, which unlike their endogenous counterparts, are localized in the nuclei of ERK1/2-transfected cells both before and after mitogenic stimulation (Wolf et al., 2001). The reason for the differences in ERK1/2 is probably the saturation of cytosolic anchoring proteins by the endogenous ERK1/2, which does not allow binding and retention of the overexpressed proteins, and therefore they move into the nucleus by a specific shuttling system (Wolf et al., 2001). Therefore, a possible explanation for the cytosolic localization of the exogenous ERK5 as oppose to the nuclear localization of the endogenous protein, could be

the saturation of nuclear binding moieties of ERK5. Thus, the inability of the exogenous protein to be retained in the nucleus is likely to promote shuttling of ERK5 into the cytosol. Indeed, our results indicate that ERK5 is able to freely shuttle in and out of the nucleus, as it is shown (Fig. 5) that exogenous ERK5 accumulates in the nucleus upon treatment of the cells with LMB. However, when in the nucleus, the exogenous ERK5 was unable to bind the nuclear anchoring moieties, as we found that NP-40 can extract the nuclear GFP-ERK5 (Fig. 5). Interestingly, the shuttling that is promoted by the lack of binding of the exogenous ERK5 to the nuclear moieties does not result in a diffuse staining, as would be expected from lack of cellular anchoring, but rather in a cytosolic staining. This suggests that the exogenous ERK5 may bind to a cytosolic set of anchoring proteins, which is completely distinct from the nuclear anchoring moieties. In line with this assumption it was shown that overexpressed N-terminal region (1-400) is localized in the cytosol (Yan et al., 2001). However, the binding to the cytosolic anchoring proteins is much more labile than to the nuclear anchoring moieties as judged from its sensitivity to NP-40 extraction (Figs 4, 5), and the fact that it is reversible after LMB treatment (Fig. 5). This is in contrast to the binding of the endogenous ERK5 in the nucleus that is NP-40 resistant, both before and after stimulation (Fig. 4). Taken together, these results support the notion that in resting cells, ERK5 is localized mainly in the nucleus because of its nuclear localization signal. In the nucleus, ERK5 is strongly attached to special anchoring moieties both before and after stimulation, and this allows the execution of its nuclear functions. When excess ERK5 is produced, the protein shifts to the cytosol, were it is bound to a labile set of anchoring proteins, and can be detached from these anchors upon persistent stimulation. Although the excess production of ERK5 shown here is of an exogenous protein, it is likely that excess of endogenous ERK5 can occur under condition that require ERK5 function in the cytosol. Under these conditions the excess of the ERK5 molecules can be retained in the cytosol and execute the relatively small number of tasks in this location (Cameron et al., 2003; Hayashi et al., 2001).

The fact that ERK5 is localized in the nuclei of both resting and activated cells raised the question as to what is the mechanism of activation of the nuclear protein. Interestingly, we found that unlike the cytosolic localization of MEK1/2 (Rubinfeld et al., 1999), MEK5 is localized in the nucleus of HeLa and Rat-1 cells both before and after stimulation (Fig. 6). In situ NP-40 extraction experiments revealed that in resting cells the MEK5 is strongly bound to the nucleus, which might be the result of strong interaction with the nuclear ERK5 or other anchoring proteins (Zhou et al., 1995). However, upon stimulation, MEK5 loses its resistance to the NP-40 treatment indicating that, similarly to ERK1/2-MEK1/2 (Wolf et al., 2001), MEK5 and ERK5 are detached from each other. However, unlike MEK1/2 that changes its location upon the detachment from MEK1/2, MEK5 is not exported out of the nucleus (Fig. 6), probably because of lack of a nuclear export signal in its sequence. The purpose of the detachment of MEK5 from its nuclear anchoring moieties upon stimulation is not known, but might contribute to signal amplification, where one molecule of MEK5 is able to phosphorylate several ERK5 molecules, all occurring within the nuclear compartment.

Although MEK5 and ERK5 are constantly localized in the nucleus, their upstream activator MEKK2 seems to transiently translocate from the cytosol into the nucleus upon mitogenic stimulation. Thus, in resting cells, MEKK2 is localized all over the cell, and in Rat-1 cells can be detected also in focal contacts (Fig. 9 and data not shown). Stimulation of the cells with EGF (Fig. 8) or treatment with LMB (Fig. 9) caused enrichment in the nuclear localization of MEKK2. Therefore, these findings support the notion that MEKK2 is the enzyme that takes the mitogenic signal of the ERK5 cascade across the nuclear envelope, and thereby activates MEK5 and ERK5. However, it is possible that MEKK2 is not the only protein that participates in the transmission of the signal to the nucleus, since other protein kinases in the MAP3K level of the ERK5 (e.g. Cot), may be involved in the transmission of the ERK5 signal as well (Chiariello et al., 2000).

In summary, we show here the subcellular localization of the components of the ERK5 signaling cascade. Our results demonstrate that MEKK2 is localized all over the resting HeLa and Rat-1 cells, and is transiently accumulated in the nucleus upon mitogenic stimulation. Thus, MEKK2 is able to transmit the signals of the ERK5 cascade into the nucleus, which is the constant site of colocalization of the other components of the cascades, MEK5 and ERK5 in both HeLa and Rat-1 cells. Its nuclear localization may assist ERK5 in executing its multiple nuclear functions. Interestingly, this subcellular localization is different from that of components of the ERK1/2 cascade, indicating that each MAPK signaling cascade may use a distinct mode of regulation, which is manifested in the distinct subcellular localization of its components.

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