Identification of a Cytoplasmic-Retention Sequence in ERK2*

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A key step in the signaling mechanism of the mitogen-activated protein kinase/extracellular signal-responsive kinase (ERK) cascade is its translocation into the nucleus where it regulates transcription and other nuclear processes. In an attempt to characterize the subcellular localization of ERK2, we fused it to the 3’-end of the gene expressing green fluorescent protein (GFP), resulting in a GFP-ERK2 protein. The expression of this construct in CHO cells resulted in a nuclear localization of the GFP-ERK2 protein. However, coexpression of the GFP-ERK2 with its upstream activator, MEK1, resulted in a cytosolic retention of the GFP-ERK2, which was the result of its association with MEK1, and was reversed upon stimulation. We then examined the role of the C-terminal region of ERK2 in its subcellular localization. Substitution of residues 312–319 of GFP-ERK2 to alanine residues prevented the cytosolic retention of ERK2 as well as its association with MEK1, without affecting its activity. Most important for the cytosolic retention are three acidic amino acids at positions 316, 319, and 320 of ERK2. Substitution of residues 321–327 to alanines impaired the nuclear translocation of ERK2 upon mitogenic stimulation. Thus, we conclude that residues 312–320 of ERK2 are responsible for its cytosolic retention, and residues 321–327 play a role in the mechanism of ERK2 nuclear translocation.

Mitogen-activated protein kinase (MAPK) signaling cascades are main routes of communication between the plasma membrane and regulatory intracellular targets and thus initiate a large array of cellular responses (1–4). The first MAPK cascade elucidated is the one that signals through the extracellular signal-responsive kinases 1 and 2 (ERK1/2), which are activated via a sequential phosphorylation and activation of the protein kinases Raf1 and MAPK/ERK kinase (MEK). Upon activation, ERK phosphorylates and activates several regulatory targets, which eventually culminate in regulation of proliferation, differentiation, and other cellular processes. Key steps in the signaling mechanism of the ERK cascade are the changes in localization of its components upon stimulation. In resting cells, all components of the cascade seem to be localized primarily in the cell cytosol. However, this localization is rapidly changed upon extracellular stimulation, which causes Raf1 recruitment to the plasma membrane (5) and translocation of MEK (6), ERK (7), and RSK (7) into the nucleus. After translocation, MEK seems to be rapidly exported from the nucleus by its nuclear export signal (NES; Ref. 8), although the timing and role of its translocation are still controversial (9, 10). ERK and RSK on the other hand are retained in the nucleus for longer times after stimulation, and this longer time is correlated with the effects of ERK on mitogenesis and neurite outgrowth in PC12 cells (11, 12).

The mechanism of nuclear translocation of the different kinases is not fully understood. Recently, it was shown that in resting cells ERK is retained in the cytosol by its association with MEK (10), and upon stimulation ERK is detached from this cytosolic anchor to rapidly translocate into the nucleus. This translocation requires dimerization and phosphate incorporation into the regulatory Thr and Tyr residues of at least one of the ERKs in the dimer (13). Here we further support the cytosolic retention of mammalian ERK2 by MEK1, which is reversed upon stimulation. We find that residues 312–320 of ERK2 play a crucial role in its MEK1-induced cytosolic retention, and thus prevent nuclear localization of ERK in resting cells. We also provide evidence that residues 321–327 play a role in the nuclear translocation of ERK2. Thus, the 312–327 region of ERK2 plays an important role in securing the proper subcellular localization of ERK2 both before and after stimulation.

EXPERIMENTAL PROCEDURES

DNA Constructs—Wild type ERK2 (bases 22-1096) was ligated into Apal and XbaI sites downstream to green fluorescent protein (GFP) gene of pGFP-C1 (CLONTECH). Mutations in ERK2 (GFP-312A, GFP-316A, GFP-321A, GFP-328A, GFP-329A, GFP-330A, and GFP-331A) were created by PCR mutagenesis as described (14). Wild type human MEK1 (MKK1) was ligated into BamHI and EcoRV sites of pCDNA1 (Invitrogen) as described (6).

Transfection and Localization Studies—The various plasmids were transfected into CHO cells using LipofectAMINE (Life Technologies, Inc.). Immunofluorescence studies were performed essentially as described (6). Cells were stimulated with peroxynitrate (VOOH; 100 μM Na3VO4/200 μM H2O2, 15 min), fixed (3% paraformaldehyde), and visualized by fluorescence microscopy (Zeiss Axioscope microscope, HCO 100 W/2, ×400 magnification). For HA-ERKs, cells were stained with anti-HA polyclonal antibody (1:100, Santa Cruz Biotechnology), and rhodamine-conjugated goat-anti-rabbit antibodies (1:100, Jackson ImmunoResearch).

Western Blotting—Transfected cells were serum-starved, stimulated, and lysed as described previously (6). Activated ERK was detected by probing blots with anti-activated ERK monoclonal antibody (1:30,000, EP ERK, Sigma Israel). Total ERK protein was detected using either anti-ERK2 C-terminal antibodies (C-14, Santa Cruz Biotechnology) or rho-DP ERK, Sigma Israel). Total ERK protein was detected using either anti-ERK2 C-terminal antibodies (C-14, Santa Cruz Biotechnology) or rho-DP ERK, Sigma Israel). MEK1 was detected with anti-MEK1 monoclonal (Transduction Laboratories).

Immunoprecipitation—Transfected CHO cells were serum-starved (24 h), stimulated (VOOH, 15 min), and harvested as described (6). The cell extracts were then subjected to immunoprecipitation with anti-GFP monoclonal antibody (1:30,000, DP ERK, Sigma Israel). Total ERK protein was detected using either anti-ERK2 C-terminal antibodies (C-14, Santa Cruz Biotechnology) or anti-MAPK (Sigma, Israel). MEK1 was detected with anti-MEK1 monoclonal (Transduction Laboratories).

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RESULTS

In an attempt to characterize the subcellular localization of ERK2, we fused the cDNA of rat ERK2 to the 3'-end of the gene expressing GFP, resulting in a GFP-ERK2 protein. This fused protein was transfected into CHO cells and was examined for its subcellular localization using fluorescence microscopy. After serum deprivation that ensures the cytosolic distribution of endogenous ERK1 and ERK2 (data not shown), GFP-ERK2 was localized primarily in the nucleus (Fig. 2). As reported for Xenopus MAPK (10), when MEK was coexpressed with GFP-ERK2, the subcellular distribution of GFP-ERK2 was different and appeared primarily in the cytosol. After stimulation with VOOH, a strong and non-selective activator of ERK, a large portion of GFP-ERK2 was translocated into the nucleus. Nuclear translocation was observed also with other stimuli (data not shown), although, a small portion (<15%) of GFP-ERK2 was observed in the cytosol under all conditions as described previously (15).

Because the C-terminal region of ERK2 plays an important role in the localization and translocation of ERK2 (13, 16), we modified several parts in this region (Fig. 1), which include (i) deletion of the C terminus of ERK2 (amino acids 338–358, ΔCOOH), (ii) substitution of amino acids 328–336 to alanines (328A), (iii) substitution of amino acids 321–327 to alanines (321A), (iv) substitution of amino acids 312–319 to alanines (312A), and (v) substitution of Asp-316, Asp-319, and Glu-320 to alanines (321A). As described for the GFP-ERK2, these constructs were fused to GFP and expressed in CHO cells, either with or without exogenous MEK1. The localization of ΔCOOH and GFP-328A appeared similar to that of GFP-ERK2 under all conditions and treatment used (data not shown). No changes as compared with GFP-ERK2 were detected also when the other three constructs 321A, 316A, and 321A, were expressed in CHO cells without additional MEK1 (Fig. 2). On the other hand, changes in localization were detected when these constructs were coexpressed with exogenous MEK1. Thus, unlike GFP-ERK2, GFP-312A was localized in the nucleus of resting cells, and this remained unchanged after VOOH stimulation. Nuclear localization was observed also with the GFP-316A protein, although the amount of this protein in the cytosol of resting cells was higher than that of the GFP-312A construct. GFP-321A had similar localization in resting cells as GFP-ERK2 when coexpressed with MEK1. However, stimulation with VOOH caused a uniform cellular distribution of this protein, indicating that this mutated protein might be compromised in its ability to translocate into the nucleus. To exclude the possibility that the altered localization is because of the GFP part of the constructs, we determined the localization of HA-ERK2 and HA-312A by staining with anti-HA antibody. Indeed, both constructs demonstrated similar distribution to that of their GFP counterparts (Fig. 2B, left), indicating that the localization observed is indeed because of ERK2. Interestingly, when the amount of transfected GFP-ERK2 was significantly reduced, the weak staining observed was either cytosolic or spread all over the cell, even in the absence of exogenous MEK1 (Fig. 2B, right). This cytosolic retention can be explained by an interaction of GFP-ERK2 with residual, unsaturated, cytosolic anchoring protein. Under these conditions, GFP-312A was still detected in the nucleus, indicating that the 312–319 region is responsible for this interaction.

The subcellular localization of the various GFP-ERK-mutants was further verified by isolating the nuclei of the GFP-ERK2 constructs-expressing CHO cells. As expected from the results in Fig. 2, Western blot analysis using anti-GFP antibodies revealed that the amount of GFP-ERK2 in the nucleus was reduced when coexpressed with MEK1 and elevated again upon stimulation (Fig. 3). The amount of GFP-312A and GFP-316A proteins in the nucleus was not significantly changed when MEK1 was coexpressed, whereas the amount of GFP-321A protein was reduced by MEK1 and only slightly enhanced after VOOH-stimulation. Together, these results show that GFP-312A, and to a lesser extent also GFP-316A, lost their MEK1-induced, cytosolic-retention in resting cells. On the other hand, GFP-321A protein exhibited a compromised VOOH-induced nuclear-translocation. Hence, residues 312–327 play an important role in the subcellular localization of ERK2 both before and after external stimulation.

To clarify whether these differences in subcellular localization are because of altered ERK2 activation, we examined the phosphorylation state and catalytic activity of the various constructs. Thus, CHO cells were transfected with GFP-ERK2, GFP-312A, GFP-316A, and GFP-321A, either with or without MEK1. The cells were then lysed and immunoblotted with either anti-general ERK or anti-diphospho ERK antibodies. Beside the endogenous ERKs, an additional band of 70 kDa was detected by the antibodies, which represents the GFP-ERKs. Similar levels of expression of the various mutated proteins were detected by the anti-general ERK antibodies (Fig. 4A). Interestingly, no significant differences in the amount of phosphates incorporated into the regulatory Thr and Tyr residues were detected between the various mutants under
any of the conditions used, indicating that the four GFP proteins undergo proper upstream activation. We then examined whether the various mutations affected the catalytic activity of ERK2. To do so, the various GFP-conjugated ERKs were immunoprecipitated with anti-GFP antibody and assayed for their ability to phosphorylate myelin basic protein (MBP). The activity of the mutants did not differ from that of GFP-ERK2, either with or without exogenous MEK1 (Fig. 4B). Therefore, these results indicate that residues 312–327 are not obligatory for either activation or catalytic activity of ERK2, and exclude the possibility that the altered localization of the modified proteins is because of compromised activation.

Because the cytosolic localization of GFP-ERK2 seems to be MEK-dependent, we next asked whether this localization is because of an association between ERK and MEK and whether the mutations in the C terminus interfere with this ERK-MEK association. Lysates from CHO cells coexpressing MEK1 and the four GFP-ERK constructs were subjected to immunoprecipitation with anti-GFP antibodies. As expected (10), immunoblotting with anti-MEK1 antibodies showed coprecipitation of MEK1 with GFP-ERK2, which was reversed upon VOOH stimulation (Fig. 5). However, this coprecipitation was not stable and was easily disrupted, even under mild washing conditions (e.g., 0.15 M NaCl; data not shown). These results support the notion that, in resting cells, ERK and MEK are indirectly associated and dissociate upon stimulation to allow translocation of both MEK and ERK into the nucleus. On the other hand, no coprecipitation of MEK was detected in immunoprecipitates of GFP-312A either before or after stimulation. When GFP-316A was immunoprecipitated from resting cells, a small amount of MEK1 was coprecipitated, in agreement with the small amount of GFP-316A that remained in the cytosol in the fluorescence studies (Fig. 2). As with GFP-ERK2, VOOH reversed the coimmunoprecipitation of MEK by GFP-316A. GFP-321A pulled down MEK1 from resting cells and less from VOOH-stimulated cells, indicating that the association and dissociation of this mutant from MEK1 were not affected. As expected, both GFP-ERK2 and GFP-312A did not interact with a constitutively active MEK1 (Fig. 5B), indicating that the activation of these cells by active MEK was sufficient to prevent the ERK-MEK association.

**DISCUSSION**

The mechanism by which ERKs are retained in the cytosol of resting cells and translocated into the nucleus is not fully.
were reproduced three times. Pressing GFP-ERK2 and MEK1 was separated by SDS-PAGE without anti-MEK and monoclonal anti-GFP antibodies. Lysate from cells expressing constitutively active MEK (N-EE-MEK; Ref. 6) together with GFP-ERK2 or GFP-312A (V00H) indicates the position of MEK1. Ab, CHO cells were transfected with either MEK1 alone (NO ERK), MEK1 with GFP-ERK2, or MEK1 with GFP-312A. After starvation, the cells were either left untreated (−) or stimulated with V00H (+). Cells were also transfected with plasmid expressing constitutively active MEK (ΔN-EE-MEK, Ref. 6) together with GFP-ERK2 or GFP-312A (CM). After immunoprecipitation with monoclonal anti-GFP, samples were immunoblotted with monoclonal anti-MEK and monoclonal anti-GFP antibodies. GFP-ERK2 indicates the position of GFP-ERK2, Ab indicates the location of the heavy chain of the antibodies used for immunoprecipitation, and MEK indicates the position of MEK1. B, CHO cells were transfected with either MEK1 alone (NO ERK), MEK1 with GFP-ERK2, or MEK1 with GFP-312A. After starvation, the cells were either left untreated (−) or stimulated with V00H (+). Cells were also transfected with plasmid expressing constitutively active MEK (ΔN-EE-MEK, Ref. 6) together with GFP-ERK2 or GFP-312A (CM). After immunoprecipitation with monoclonal anti-GFP, samples were immunoblotted with monoclonal anti-MEK and monoclonal anti-GFP antibodies. Lysate from cells expressing GFP-ERK2 and MEK1 was separated by SDS-PAGE without prior immunoprecipitation as a control (Extract). These experiments were reproduced three times.

understood. Based on our and others studies (10, 16), it seems that ERK is kept out of the nucleus of resting cells by several anchoring proteins. One docking matrix is the microtubule cytoskeleton, which serves as a major docking matrix for up to 35% of the cellular ERK1 and ERK2 (15). Other putative anchoring proteins for ERK in the cytosol are MAP kinase phosphatases (MKPs) and in particular MKP3 (17, 18), but these interactions do not seem to be reversed by mitogenic stimulations.

Unlike the activity-independent binding of ERK to microtubules and MKPs, our results indicate that the cytosolic retention of ERK2 by MEK1 is reversed by mitogenic stimulation, thus allowing the nuclear translocation of ERK. Similar results were previously obtained for Xenopus ERK (10), and we noticed the same also with ERK1 (data not shown). However, the amount of endogenous MEK1 seems to be significantly lower than that of ERKs in many cells (20) and cannot account for the cytosolic retention of all ERKs. Moreover, ERK and MEK association is easily disrupted, and a small amount of ERK can be retained in the cytosol without exogenous MEK. It is therefore possible that additional proteins (e.g., the scaffold protein MP1 (21)) may allow free movement of ERK in the cytosol, thus forming big signaling complexes, which may include more than one ERK molecule.

In this study we examined the role of the C-terminal region of ERK in determining its subcellular localization. We found that deletion of the C-terminal residues 338–358 or substitution of residues 328–336 to alanines did not affect the subcellular localization of ERK2. However, it was reported (13) that this combined C-terminal region (residues 328–358) contains a putative NES and dimerization-promoting leucine residues. A possible explanation for the lack of influence of the mutations is that each one of the mutations covers only part of the dimerization residues or the NES and the truncated parts are still active.

In contrast to residues 328–358, mutations in residues 321–327 affected nuclear translocation, and residues 312–320 affected cytosolic retention of ERK. The most important residues for the cytosolic retention seem to be the three acidic residues Asp-316, Asp-319, and Glu-320. However, because the effect of GFP-316A was weaker than that obtained with GFP-312A, it is likely that additional residues between 321–318 contribute to the cytosolic retention. Interestingly, Asp-319, which is substituted to Gln in the Drosophila sevenmaker gain-of-function mutant, was found to be responsible also for the binding of ERK to MKPs (18). However, because MKPs are inducible proteins (19) and are not expressed in any significant amount in resting CHO cells (data not shown), this association is unlikely to account for the cytosolic retention of ERK in resting cells.

Upon mitogenic stimulation, ERK (7) as well as MEK (6, 22) are activated and translocate into the nucleus. According to our results, this process involves dissociation of these two kinases. It is likely that after dissociation from the anchoring protein, ERK passively diffuses to the nuclear membrane and then penetrates to the nucleus by an unknown mechanism. The molecular size of the endogenous ERK and MEK (<50 kDa) may allow free diffusion into the nucleus. However, it was suggested that ERK undergoes a phosphorylation-dependent dimerization, which allows its proper translocation (13), and we show here that the 70-kDa GFP-ERK2 can rapidly translocate into the nucleus. Thus, it is likely that the molecular size of these proteins and the fast kinetic of translocation of the endogenous ERK requires an active mechanism, which might involve interactions with importins or exportins, to cross the nuclear membrane.

In summary, we found that substitution of residues 312–319 of ERK2 with alanine residues does not change its activity, but prevents the cytosolic retention of ERK, as well as its association with MEK1. Substitution of residues 321–327 to alanines impairs the nuclear translocation of ERK2 upon mitogenic stimulation. We conclude that residues 312–327 of ERK2 play a role in its subcellular localization both before and after stimulation.

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