Different Domains of the Mitogen-activated Protein Kinases ERK3 and ERK2 Direct Subcellular Localization and Upstream Specificity in Vivo*

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The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated protein kinase; MEK, MAP kinase/ERK kinase; MBP, myelin basic protein; ERK3αC, the catalytic domain of ERK3, which lacks the C-terminal 180 amino acids from the 63-kDa form; D, docking; CD, common docking; GST, glutathione S-transferase.

Extracellular signal-regulated kinase 3 (ERK3) is a member of the mitogen-activated protein (MAP) kinase family. ERK3 is most similar in its kinase catalytic domain to ERK2, yet it displays many unique properties. Among these, unlike ERK2, which translocates to the nucleus following activation, ERK3 is constitutively localized to the nucleus, despite the lack of a defined nuclear localization sequence. We created two chimeras between ERK2 and the catalytic domain of ERK3 (ERK3αC), and some mutants of these chimeras, to examine the basis for the different behaviors of these two MAP kinase family members. We find the following: 1) the N-terminal folding domain of ERK3 functions in phosphoryl transfer reactions with the C-terminal folding domain of ERK2; 2) the C-terminal halves of ERK2 and ERK3αC are primarily responsible for their subcellular localization in resting cells; and 3) the N-terminal folding domain of ERK2 is required for its activation in cells, its interaction with MEK1, and its accumulation in the nucleus.

Nearly all cells respond to extracellular stimuli by activation of one or more members of the ubiquitous mitogen-activated protein (MAP) kinase family. Activation of MAP kinases produces cell type- and ligand-specific responses. MAP kinases are regulated by a cascade composed of a three-kinase module in which a MAP/ERK kinase (MEKK or MAP3K) activates a MEK (also known as MKK or MAP2K), the dual specificity kinase that activates the MAP kinase. In yeast systems, five MAP kinase pathways have been described (5); more than a dozen MAP kinases are known in mammalian cells. The best characterized MAP kinase module contains the mitogen-sensitive ERK1 and ERK2, and a second module contains the related protein ERK5. Other well-characterized modules contain the stress-sensitive c-Jun N-terminal kinase/stress-activated protein kinases and p38 MAP kinases, which respond to inflammatory cytokines and osmotic shock, among other stresses (1, 3, 4, 7).

Certain MAP kinases, including ERK3 and ERK7, are considered orphans because their upstream regulatory mechanisms are poorly understood (8–13). Interestingly, ERK3 homologs are not present in budding yeast or the nematode C. elegans, indicating that ERK3 is a relatively late addition to the metazoan signaling repertoire. A cDNA encoding ERK3 was cloned 10 years ago using an ERK1-derived cDNA probe (14).

Several key properties of ERK3 set it apart from the classical MAP kinases, despite the fact that ERK3 is ~50% identical to ERK1 and ERK2 in its catalytic core, making ERK3 one of their closest relatives. First, ERK3 contains a long C-terminal extension. Proteins of 63 and 95–100 kDa have been reported and are represented by cDNAs from several species that probably are derived from two distinct genes (9, 14–16). In ERK5, which also contains a C-terminal domain following the kinase catalytic core, the extension may harbor an autoinhibitory motif; however, to date no function for the ERK3 C-terminal extension has been described, and its removal does not affect the few properties of the enzyme that are known (8, 17). Second, ERK3 is unique among the MAP kinases in that it contains only a single phosphorylation site (serine 189) in its activation loop. The other MAP kinases have the sequence TXY in the loop, but ERK3 contains SEG, with glycine in place of the tyrosine. In ERK2 phosphorylation of tyrosine in this loop causes a refolding of the protein substrate-binding site and is required for the observed kinetic and threshold behavior of its cascade (18–20). This distinction implies that activation by upstream regulators and interactions with substrates are likely to occur differently with ERK3 than with other family members. Consistent with these suggestions, none of the currently known MEK family members is able to phosphorylate and activate ERK3, and no physiological substrates of ERK3, other than itself, have yet been found. An ERK3 kinase activity has been partially purified that recognizes ERK3 alone of the known MAP kinases, phosphorylating it on serine 189 in the activation loop (8). Because ERK3 does not phosphorylate other substrates tested, it has not been possible to determine whether phosphorylation increases its activity. Finally, ERK3 is localized constitutively to the nucleus despite the lack of a traditional nuclear localization sequence. This is in contrast to ERK1 and ERK2, which translocate to the nucleus upon phosphorylation by MEKs via a mechanism that may, at least in the case of ERK2, be enhanced by the formation of kinase dimers (21, 22).

To examine the basis for differences in these two MAP kinase family members, we made two chimeric proteins between ERK2 and ERK3. One chimera contained the N-terminal folding domain of ERK3 and the C-terminal folding domain of...
ERK2, and the other contained the N-terminal folding domain of ERK2 and the rest of the catalytic domain of ERK3. In addition, we exchanged the ERK2 and ERK3 residues in the common docking motif, a region of MAP kinases believed to interact with the docking (D) motif of MEKs and other proteins. We find that the C-terminal portion of the catalytic core is significantly responsible for localization of the kinases. However, the chimera containing the C terminus of ERK2, although found in the cytoplasm and nucleus of resting cells, can no longer accumulate in the nucleus upon activation nor is it exported from the nucleus by overexpression of MEK1. Finally, although either of the two folding domains of the kinase core is sufficient for MEK recognition in vitro, in transfected cells the N-terminal region of ERK2 is important for its activation in a manner not revealed by in vitro assays, perhaps due to the formation of multiprotein complexes. These results will be discussed in the context of known sites of MEK1-ERK1/2 interaction (23–27).

MATERIALS AND METHODS

Construction of Chimeras—The construct encoding the ERK3/2 chimeras was introduced using the unique Spel site in ERK3 at the residue His-128 in subdomain V (Fig. 1B). A rat ERK2 fragment encoding Ile-123 (located in the same region of subdomain V) through the end of ERK2 was obtained using PCR and ligated to an ERK3 fragment encoding residues 1–128. This chimera DNA and the one described below were subcloned into pGEX-KG and pCMV5/Myc for bacterial and mammalian expression, respectively. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene) according to the manufacturer’s instructions.

To generate the construct encoding ERK2/3/4C, approximately the same junction in subdomain V of the MAP kinases was used. Both fragments encoding the MAP kinases were obtained via PCR. An Neol-XhoI fragment encoding N terminus of ERK2 and an XhoI-HindIII fragment encoding the C-terminal half of the catalytic domain of ERK3 were ligated to create the second chimera. Amino acid changes resulting from the addition of the new restriction sites are shown in Fig. 1B. The aspartate to alanine and CD motif mutations were introduced with the QuikChange double-stranded DNA mutagenesis kit (Stratagene, La Jolla, CA) and were confirmed by DNA sequencing.

Protein Expression and Purification—BL21-DE3pLys bacteria containing GST fusion protein plasmids were grown in Terrific Broth at 30 °C to an A600 of ~0.6–0.8 and then induced with 100 μM isopropyl-1-thio-β-D-galactopyranoside overnight. Proteins were purified from lysates over glutathione-agarose as described (28, 29). Phosphorylated and active MEK1, MEK2, and MEK3 were purified as described previously (30). MEK4 was activated by a catalytic fragment of MEK1 as described previously (29), MEK6, which has high basal activity, was used without activation.

Protein Kinase Assays—In vitro kinase assays were performed as described previously (29). To measure ERK3 kinase activity, either partially purified ERK3 kinase or pig brain cytosol (gift of P. Sternweis, University of Texas Southwestern) was added to glutathione-agarose beads previously bound to GST fused to ERK3 (8), ERK2, ERK3/2, or ERK2/3/4C. Samples were rocked at 4 °C for 2 h. Beads were then washed with 1× NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% Triton X-100, 1 mM EDTA, and once with kinase buffer (see below). Under these conditions, the ERK3 kinase remains tightly bound to the ERK3 on the beads (8). Phosphorylation of the GST fusion proteins was initiated by addition of MgATP trace-labeled with [γ-32P]ATP to the beads, followed by incubation at 30 °C for 1 h, and analyzed by SDS–PAGE and autoradiography as described (8).

Mammalian Cell Culture and Transfection—HEK293 cells were transfected using calcium-phosphate (31). 10 μg of ERK chimera plasmids were used per 60-mm plate unless otherwise indicated. Lysates from the cells that were serum-starved for 24 h were prepared 48 h after transfection. For the ERK2/3/4C chimera, lysates were prepared in RIPA buffer (29) to better solubilize the protein. For all other proteins, lysates were prepared in 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40.

Western Blotting and Immunoprecipitation—The anti-Myc antibody was from the Cell Culture Center and was used at 1:100 for blotting. The anti-hemagglutinin antibody 12CA5 (Babco, Richmond, CA) was used for Rsk immunoprecipitations and blotting. The anti-phospho-ERK2 monoclonal antibody was purchased from New England Biolabs and was used as suggested by the manufacturer. All immunoprecipitations and Western blotting were done as described previously (32). HA-Rsk was detected using the substrate GST-S6 as previously described (32). Both constructs were the generous gift of J. Blenis (Harvard University).

Immunofluorescence—HEK293 cells were plated on polylysine-coated coverslips and transfected as above. Cells were fixed in 3.7% formaldehyde for 5 min and were permeabilized in 0.1% Triton X-100 for 10 min at room temperature. After washing, slips were incubated in anti-Myc antibody (1:100) for 1 h at 37 °C and in anti-mouse IgG fluorescein isothiocyanate-conjugated antibody (1:2000) for 30 min at 37 °C. To visualize the nucleus, slips were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole for 2 min at room temperature.

RESULTS

Design of the Chimeric Proteins—The two chimeras of ERK2 and ERK3 and the mutants in which residues from the CD motifs have been exchanged are diagrammed in Fig. 1A. One chimera, ERK3/2/3, contains the N-terminal half of the ERK3 catalytic core, subdomains I–V, and the C-terminal half of the ERK2 catalytic core, subdomain V through the end of the ERK2 coding sequence. The second chimera, ERK2/3/4C, contains subdomains I–V of ERK2 and the rest of the catalytic domain of ERK3, but lacks the C-terminal 180 residues of the 63-kDa form of ERK3. Deletion of these residues has a negligible effect on the subcellular localization of ERK3 or its binding to or phosphorylation by a partially purified ERK3 kinase (8, 17). The sequences at the junctions of the two domains are shown in
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**Table I. Properties of chimeric proteins**

| Property | ERK2 | ERK3ΔC | ERK3/2 | ERK2/3ΔC |
|----------|------|--------|--------|----------|
| Phosphorylated by MEK1, -2 in vitro | Yes | No | Yes | No |
| Phosphorylated by ERK3 kinase in vitro | No | Yes | No | Yes |
| Bound to ERK3 kinase | No | Yes | Yes | Yes |
| Activated by MEK1 | Yes | — | Yes | — |
| Activated by MEKR4F | Yes | — | No | — |
| Activated by Raf1Xb | Yes | — | No | — |
| Activated by RasV12 | Yes | — | No | — |
| Resting localization | Nucleus | Nucleus | Througout cell | Nucleus |
| Stimulated localization | Nucleus | Nucleus | Througout cell | Nucleus |
| Localization with overexpressed MEK1 | Cytoplasm | — | Yes | — |
| Rsk activation | Yes | — | Yes | — |
| ERK2 CD site present | Yes | No | Yes | No |

*a* — not tested.

*b* Shown previously (24, 26).

Fig. 1B, and the sequences encompassing the CD motifs are indicated in Fig. 1C with the conserved residues in bold and the residues that were exchanged underlined (Tyr-315 and Asp-316 of ERK2 exchanged for Ser-320 and Phe-321, respectively, the comparable positions of ERK3). The properties of these chimeras are summarized in Table I.

**In Vitro Phosphorylation and Kinase Activity of the Chimeric Proteins**—The two chimeras were expressed as GST fusion proteins in bacteria and were the predicted size (Fig. 1D). A doublet was often observed with ERK2/3ΔC; both bands cross-reacted with anti-GST and anti-ERK3 antibodies (data not shown).

ERK2 and ERK3 are not phosphorylated by the same upstream kinases (8). Therefore, we tested the ability of the two chimeras to be phosphorylated by MEK1, MEK2, and the ERK3 kinase in vitro to ascertain if specificity-determining regions could be mapped to either half of the catalytic domain. Brunet and Pouysségur (33) suggested that MAP kinase subdomains I–V, the N-terminal half of its catalytic core, determine the upstream MEKs that will phosphorylate a MAP kinase in cells. If this is the case in vitro, the ERK3/2 chimera should not be recognized by the ERK2-selective enzymes MEK1 and MEK2. Because it contains the protein substrate-bindig site, ERK3/2 should phosphorylate ERK2 substrates. As shown in Fig. 2A, ERK3/2 was phosphorylated by MEK1 and MEK2 with a stoichiometry similar to that for ERK2 (29). Both tyrosine and threonine were phosphorylated (Fig. 2B), and the rate of phosphorylation was only slightly slower than that for ERK2 (Fig. 2C). Like ERK2, ERK3/2 was not phosphorylated by MEK3, -4, or -6 (data not shown).

After phosphorylation, the ERK2/3 chimera displayed a specific activity of ~200 nmol/min/mg with myelin basic protein (MBP) as substrate (Fig. 2D); this is more than a 100-fold increase in activity compared with unphosphorylated protein and about 10–20% of the value for wild type-phosphorylated ERK2. Because the stoichiometries of ERK3/2 phosphorylation by MEK1 and MEK2 were not significantly different from those of ERK2, the lower MBP kinase activity is most likely due to minor changes in folding of the chimera in the active site. No substrates of ERK3 other than itself have been identified; thus, there has been a question about its capacity to catalyze phosphoryl transfer. The relatively high activity of the chimera containing the N-terminal domain of ERK3 indicates that ERK3 can interact with ATP in a manner appropriate for catalysis.

The ERK2/3ΔC chimera was phosphorylated poorly by all the MEKs but was reproducibly phosphorylated to a small extent by MEK2 (Fig. 2A), consistent with the weak phosphorylation of ERK3 by MEK2 and its lack of phosphorylation by other known MEK family members (29). Also consistent with earlier experiments, no detectable increase in kinase activity could be measured using MBP (data not shown).

**Binding and Phosphorylation of Chimeras by the ERK3 Kinase**—Previously, we partially purified an activity that bound to ERK3 (and ERK 3ΔC) and phosphorylated it on serine 189. This activity did not bind or phosphorylate other MAP kinases (8). Therefore, we determined whether this ERK3 kinase could bind and phosphorylate either of the chimeric proteins. The two chimeras were attached to glutathione beads and incubated with pig brain cytosol containing ERK3 kinase activity. The ERK3 kinase bound to both chimeras, as assessed by the ability of the activity on the beads to phosphorylate exogenously added full-length ERK3 (see below and data not shown). The ERK3 kinase also phosphorylated ERK2/3ΔC (Fig. 3A). However, the extent of chimera phosphorylation was much less than that of ERK3 itself (Fig. 3B). When serine 189 was mutated to alanine, the ERK3 kinase no longer phosphorylated ERK3ΔC, consistent with its previously characterized specificity (8). ERK2 neither binds nor is phosphorylated by the ERK3 kinase (8). Despite the fact that the ERK3 chimera bound to the ERK3/2 chimera, this chimera was not phosphorylated by the ERK3 kinase (Fig. 3B), supporting the previously suggested distinction in the ability to bind and the inherent enzymatic specificity of this kinase. These results indicate that the ERK3 kinase interacts strongly with both domains of ERK3.

**Phosphorylation and Activation of Chimeras in 293 Cells**—The in vitro results suggested that the C-terminal domain of ERK2 is sufficient for recognition by MEK1 and -2, consistent with the presence of the common docking (CD) motif in this domain (23, 24, 34). The CD motif is involved in binding MEK1, substrates, and certain MAP kinase phosphatases (23). To examine the relationship of our findings to the work of Brunet and Pouysségur (33), who characterized regulation of ERK1-38 chimeras in transfected cells, we expressed epitope-tagged forms of ERK2, ERK3ΔC, and the chimeras in mammalian cells, and we examined their regulation by components of the ERK/MAP kinase cascade (Fig. 4). As expected, neither ERK3ΔC nor ERK2/3ΔC were detectably activated in cells by any of the agents, as measured by phosphorylation of MBP (data not shown). This is consistent with the lack of MBP kinase activity of ERK3 after phosphorylation by the ERK3 kinase (8). Activity in ERK2/3ΔC immunoprecipitates from MEK1-cotransfected cells was due to a coprecipitating kinase, based on the observation that mutation of an essential catalytic residue in ERK2/3ΔC (D of subdomain VII to A) produced an immunoprecipitate with equivalent activity.

ERK3/2 was activated only weakly in cells by coexpression with wild type MEK1 or MEK2, typical of their effects on wild type ERK2 (Fig. 4A). On the other hand, the highly active mutant form of MEK1, MEK1R4F, activated ERK3/2 nearly as
well as it activated ERK2 (Fig. 4A). Although MEK2 activated the 3/2 chimera better in vitro than did MEK1, in cells the activated MEK2 mutant, MEK2R4F, activated the 3/2 chimera much less well than MEK1R4F. We next tested the ability of more upstream components of the cascade to activate ERK3/2. Contrary to expectations, neither RafBXB, a constitutively active form of Raf, nor RasV12 activated ERK3/2 to a significant extent. Their effects were much less than those of either MEK1R4F or MEK2R4F. Thus, activators of the ERK2 pathway were not equally effective in stimulating the ERK3/2 chimera, although they had the expected effects on ERK2.

ERK3/2 also possessed the ability to activate the ERK2 substrate p90Rsk in cells. As seen in Fig. 4B, neither ERK2 nor ERK3/2 alone stimulated Rsk activity. MEK1R4F stimulated Rsk activity due to phosphorylation by endogenous ERK1/2. However, when overexpressed in the presence of MEK1R4F, both ERK2 and ERK3/2 show an equal ability to activate Rsk further. Under these conditions expression of ERK2 is generally greater than ERK3/2 (Fig. 4C). This suggests that when expressed in cells, the chimera recognizes ERK2 substrates, consistent with the presence in this chimera of the CD site localized to the C-terminal half of ERK2 (23, 24).

Subcellular Localization of Chimeras—ERK3 and ERK2 are distributed differently in cells. ERK2 is predominantly cytosolic until cell stimulation at which time it accumulates in the nucleus (17, 35–38). Multiple mechanisms may be involved in the nuclear accumulation of ERK2, including formation of complexes with other proteins that may carry it in, dimerization, and the cessation of MEK-mediated export due to the dissociation of MEK as a consequence of ERK2 phosphorylation (21, 22, 39). ERK3 is constitutively localized to the nucleus (17). The portions of ERK3 that target it to the nucleus are un-
known, although the C-terminal tail is not required (17). Thus, we investigated the localization of ERK3/2 and ERK2/3 in 293 cells to determine whether regions that confer distinct subcellular distributions could be identified.

In the absence of stimuli, ERK2 was distributed throughout the cell, primarily in the cytoplasm and to a small extent in the nucleus (Fig. 5A). As expected, in the presence of cotransfected, active MEK1R4F, ERK2 redistributed to the nucleus. ERK3/H9004C, on the other hand, was found most concentrated in the nucleus, apparently in nuclear speckles, although the speckles are not as pronounced with the high levels of expression observed in Fig. 5. As shown previously, various ligand treatments did not change this distribution (Fig. 5A (17)).

The localization of ERK2/3ΔC resembled that of ERK3 and ERK3ΔC in that it was found in nuclear speckles (Fig. 5A). In contrast to the behavior of ERK2, the nuclear localization of this chimera occurred in the absence of cotransfection with MEK1R4F and did not change in its presence. ERK3/2 was localized throughout the cell but mainly in the cytoplasm.

Thus, it resembled ERK2 in its distribution in resting cells, as noted above. Cells expressing ERK3/2 often appeared flatter, but the significance of this observation is not known. When
ERK3/2 was coexpressed with MEK1R4F, which activated it strongly in these cells (Fig. 4), there were minimal changes in its intracellular localization. It did not translocate to the nucleus in a manner comparable with ERK2.

ERK2 is thought to be exported from the nucleus by association with MEK1 (40). The best defined site of interaction of ERK2 with MEK1 is through the association of the MEK1 D domain with a region of ERK2 in its C-terminal domain that has been called the CD site. Thus, we coexpressed the chimeras with MEK1 to determine whether MEK1 might alter the localization of the chimeras (Fig. 5A). This coexpression experiment is one of the most sensitive assays of the intracellular association of ERK2 and MEK1 (24). Coexpression of wild type ERK2 with MEK1 caused the loss of ERK2 from the nucleus as demonstrated previously (24, 26). However, MEK1 did not cause relocalization of either the ERK2/3 or the ERK3/2 chimera. To determine whether the CD motif might impact these results, the chimeras were mutated to exchange two residues in the CD sites of ERK2 and ERK3. Examination of the sequence of ERK3 suggests that the CD site is well conserved, with the exception of the two residues that we chose to exchange between the two chimeras: Tyr-315 and Asp-316 of ERK2 for Ser-320 and Phe-321 of ERK3 (Fig. 1C). In ERK2 these residues contribute significantly to recognition of the MEK1 D domain (24, 26). The 3/2 and 2/3 chimeras with the exchanged CD motif residues were localized in a manner indistinguishable from the 3/2 and 2/3 chimeras. We might have expected that exchanging these residues would have enhanced the likelihood that MEK1 would bind to the mutant 2/3 chimera. In contrast, coexpression with MEK1 had no impact on the localization of either the 3/2 or the 2/3 chimera in which the CD motif residues had been swapped (Fig. 5B).

**DISCUSSION**

**Signaling Specificity**—Brunet and Pouysségur (33) suggested that the N-terminal domain of MAP kinases directed recognition by MEKs, based on the specificity of activation of ERK1-p38 chimeras in transfected cells by stimuli selective for the individual kinases. Our *in vitro* findings using ERK2-ERK3 chimeras indicate that the C-terminal domain of the catalytic core is sufficient for MEK recognition, because the ERK3/2 chimera was phosphorylated in a manner similar to wild type ERK2. This conclusion is supported by previous studies (23, 41, 42) on chimeras of ERK5 and p38 with ERK2, and by the identification of the CD motif in the C-terminal domain of ERK2. Although only the C-terminal half of ERK2 was necessary for recognition by ERK2-specific MEKs in our earlier chimera studies, the MEK-interacting surface was found to be contributed by both MAP kinase domains (42). The ERK3 kinase, which may be a member of the MEK family, displays a similar ability to interact with both domains of ERK3 *in vitro*.

To evaluate the importance of the intracellular environment on specificity, we examined the properties of the ERK3/2 chimera when expressed in mammalian cells. The activation pattern of the 3/2 chimera in cells was different from that *in vitro*. MEK1R4F activated ERK3/2 to a similar extent as it did ERK2 both *in vitro* and in cells. However, neither activated mutants of components further upstream in the pathway, e.g. Ras, nor ligands activated ERK3/2. These results are consistent with the idea that there is an interaction involving the N terminus of ERK2 that is required for its intracellular, although not its *in vitro*, activation and thus support the conclusion of Brunet and Pouysségur (33).

What might account for the failure of the regulatory machinery to recognize the ERK3/2 chimera in cells? One possibility is that the binding of MEK itself to the N terminus of ERK2 inside cells allows ERK2 to interact in a productive manner with the upstream cascade. Because overexpression of MEK was required to demonstrate activation of the 3/2 chimera, it could be argued that there was a defect in ERK-MEK binding that could be overcome by increasing the amount of MEK in the cell. Failure of upstream signals to activate the chimera might reflect the inability of endogenous MEK to interact properly with 3/2. Supporting the idea of a weakened MEK interaction is the observation that MEK1 overexpression does not reduce the amount of ERK3/2 in the nucleus. This suggests that the C terminus of ERK2 is not sufficient for its intracellular interaction with MEK1. We found that MEK phosphorylation of ERK2, but not of a p38-ERK2 chimera lacking the ERK2 N terminus, is reduced by deleting the ERK-docking site within the N terminus of MEK1 (25). That observation together with our current findings and a study by Weber and co-workers (27) suggest that there may be an additional site of ERK-MEK binding in the N terminus of ERK2 that is essential for intracellular ERK2 activation. A second possibility is that an interaction of the N terminus of ERK2 with a protein other than MEK is required for its activation in cells and its nuclear export in complex with MEK1.

**Subcellular Localization**—We find that the C-terminal domains of ERK2 and ERK3 are sufficient to mimic the subcellular localization of the wild type proteins in resting cells. The features of ERK3 that specify a constitutively nuclear location apparently are contained in the C-terminal half of its catalytic core. The presence of the ERK2 N terminus did not disrupt the localization nor did additionally swapping residues in the
ERK3 region comparable to the CD motif with those in ERK2. Supporting the role of the C-terminal domain in localization, the ERK3/2 chimera was found throughout the cell, as is wild type ERK2. This is consistent with recent studies (23, 24) identifying the CD motif in the C-terminal portion of ERK2 as a cytoplasmic retention sequence. However, mutating two of the key residues in the CD motif did not alter the localization of this chimera, suggesting that the localization is not due solely to the CD motif.

Based many studies, a major step in the control of ERK2 localization is its nuclear export (43). The nuclear export receptor CRM1, which binds to hydrophobic nuclear export sequences, is required. Because its association with MEK1 has a nuclear export sequence, MEK1 may be primarily responsible for the export of ERK2 from unstimulated cells (40, 44). Thus, the absence of an appropriate ERK2-MEK1 interaction may impair the nuclear export of the chimera; apparently both N and C termini of ERK2 are required for the normal ERK2-MEK1 interaction. Data with the 3/2 and 2/3 chimeras support this conclusion. Less clear is why the C-terminal half of ERK2, although apparently required, is not sufficient for its stimulus-mediated nuclear accumulation. Three studies have concluded that portions of the C terminus of ERK2 are required for nuclear accumulation. An alanine-scanning study suggested that the C terminus contained a sequence required for nuclear entry (24). Two earlier studies suggested that active import of ERK2 requires dimerization of the kinase (21, 22). ERK2 forms dimers upon phosphorylation, and the dimer interface has been mapped to the ERK2 C terminus (21). The conformational changes that sup-

REFERENCES

1. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Adv. Cancer Res. 74, 49–139
2. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) Exp. Cell Res. 253, 255–270
3. Davis, R. J. (1999) Biochem. Soc. Symp. 64, 1–12
4. Ichijo, H. (1999) Oncogene 18, 6087–6093
5. Hunter, T., and Plowman, G. D. (1997) Trends Biochem. Sci. 22, 18–22
6. Plowman, G. D., Sudarsasanam, S., Bingham, J., Whyte, D., and Hunter, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13603–13610
7. Pearson, G., Robinson, F., Beers, G. T., Xu, B., Karandikar, M., Berman, K., and Cobb, M. H. (2001) Endocr. Rev. 22, 153–183
8. Cheng, M., Zhen, E., Robinson, M. J., Ebert, D., Goldsmith, E., and Cobb, M. H. (1999) J. Biol. Chem. 274, 12057–12062
9. Zhu, A. X., Zhan, Y., Moller, D. E., and Flier, J. S. (1994) Mol. Cell. Biol. 14, 8202–8211
10. English, J. M., Pearson, G., Hockenberry, T., Shivakumar, L. W., White, A., and Cobb, M. H. (1999) J. Biol. Chem. 274, 31588–31592
11. Kamakura, S., Moriguchi, T., and Nishida, E. (1999) J. Biol. Chem. 274, 26563–26571
12. Kato, Y., Tapping, R. I., Huang, S., Watson, M. H., Ulevitch, R. J., and Lee, D. D. (1998) Nature 395, 713–716
13. Abe, M. K., Kuo, W. L., Hershenson, M. B., and Rosner, M. R. (1999) Mol. Cell. Biol. 19, 1301–1312
14. Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radezjeiwes, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., and Yanceopus, G. D. (1991) Cell 65, 663–675
15. Gonzalez, F. A., Raden, D. L., Rigby, M. R., and Davis, R. J. (1992) FEBS Lett. 304, 170–178
16. Turgeon, R., Saba-El-Leil, M. K., and Meloche, S. (2000) Biochem. J. 346, 169–175
17. Cheng, M., Boulton, T. G., and Cobb, M. H. (1997) J. Biol. Chem. 272, 8851–8858
18. Camagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. (1997) Cell 90, 859–869
19. Ferrell, J. E., and Mitchluder, E. M. (1998) Science 280, 895–898
20. Prowse, C. N., and Lee, J. (1999) J. Biol. Chem. 274, 99–103
21. Khokhlatchev, A., Camagarajah, B., Wilsbacher, J. L., Robinson, M., Atkinson, M., Goldsmith, E., and Cobb, M. H. (1998) EMBO J. 17, 5347–5358
22. Tanne, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) Nat. Cell. Biol. 2, 110–116
23. Rubin, H., Hanoch, T., and Seger, R. (1999) J. Biol. Chem. 274, 30349–30352
24. Xu, B., Wilsbacher, J. L., Collins, T., and Cobb, M. H. (1999) J. Biol. Chem. 274, 34029–34035
25. Xu, B., Sippe, S., Robinson, C., and Cobb, M. H. (2001) J. Biol. Chem. 276, 20059–20065
26. Ehlen, S. T., Callings, A. D., Assanah, M. C., and Weber, M. J. (2001) Mol. Cell. Biol. 21, 249–259
27. Guan, K., and Dixon, J. E. (1999) Anal. Biochem. 262, 262–267
28. Robinson, M. J., Cheng, M., Khokhlatchev, A., Ebert, D., Ahn, N., Guan, K., Stein, B., Goldsmith, E., and Cobb, M. H. (1996) J. Biol. Chem. 271, 29734–29739
29. Khokhlatchev, A., Xu, S., English, J., Wu, P., Schafer, E., and Cobb, M. H. (1999) J. Biol. Chem. 274, 6159–6165
30. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Robinson, M. J., Sippe, S. A., Goldsmith, E., White, A. M., and Cobb, M. H. (1998)Curr. Biol. 8, 1141–1150
32. Brunet, A., and Pouyssegur, J. (1996) Science 272, 1653–1655
33. Rognes, C., Li, P. P., Schlacht, L., Parikh, S. V., Cooke, R., and Warsh, J. (2000) Biol. Psychiatry 48, 665–673
34. Chen, R.-H., Sarnecki, C., and Blenis, J. (1999) Mol. Cell. Biol. 12, 915–927
35. Gonzalez, F. A., Seth, A., Raden, D. L., Bowman, D. S., Fay, F. S., and Davis, R. J. (1998)J. Cell Biol. 122, 1089–1103
36. Lenormand, P., Sardet, C., Pages, G., L’Allemain, G., Brunet, A., and Pouyssegur, J. (1993) J. Cell Biol. 122, 1079–1088
37. Razika, A. A., Seger, R., Dilts, C. D., Krebs, E. G., and Fischer, E. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8881–8885
38. Fukuda, M., Gotoh, Y., and Nishida, E. (1997) EMBO J. 16, 1901–1908
39. Fukuda, M., Gotoh, I., Adachi, M., Gotoh, Y., and Nishida, E. (1997) J. Biol. Chem. 272, 32642–32648
40. English, J. M., Pearson, G., Baer, R., and Cobb, M. H. (1998) J. Biol. Chem. 273, 3854–3860
41. Wilsbacher, J. L., Goldsmith, E. J., and Cobb, M. H. (1999) J. Biol. Chem. 274, 16988–16994
42. Adachi, M., Fukuda, M., and Nishida, E. (2000)J. Cell Biol. 149, 849–856
43. Fukuda, M., Gotoh, I., Gotoh, Y., and Nishida, E. (1996) J. Biol. Chem. 271, 20024–20030
44. Lenormand, P., Brondello, J. M., Brunet, A., and Pouyssegur, J. (1998)J. Cell Biol. 142, 625–633
45. Mahanty, S. K., Wang, Y., Farley, F. W., and Elion, E. A. (1999) Cell 98, 501–512

* S. Stippec, unpublished observations.