ERK2 nuclear-cytoplasmic distribution is regulated in response to hormones and cellular state without the requirement for karyopherin-mediated nuclear import. One proposed mechanism for the movement of ERK2 into the nucleus is through a direct interaction between ERK2 and nucleoporins present in the nuclear pore complex. Previous reports have attributed regulation of ERK2 localization to proteins that activate or deactivate ERK2, such as the mitogen-activated protein (MAP) kinase kinase MEK1 and MAP kinase phosphatases. Recently, a small non-catalytic protein, PEA-15, has also been demonstrated to promote a cytoplasmic ERK2 localization. We found that the MAP kinase insert in ERK2 is required for its interaction with PEA-15. Consistent with its recognition of the MAP kinase insert, PEA-15 blocked activation of ERK2 by MEK1, which also requires the MAP kinase insert to interact productively with ERK2. To determine how PEA-15 influences the localization of ERK2, we used a permeabilized cell system to examine the effect of PEA-15 on the localization of ERK2 and mutants that have lost the ability to bind PEA-15. Wild-type ERK2 was unable to enter the nucleus in the presence of an excess of PEA-15; however, ERK2 lacking the MAP kinase insert largely retained the ability to enter the nucleus. Binding assays demonstrated that PEA-15 interfered with the ability of ERK2 to bind to nucleoporins. These results suggest that PEA-15 sequesters ERK2 in the cytoplasm at least in part by interfering with its ability to interact with nucleoporins, presenting a potential paradigm for regulation of ERK2 localization.

The mitogen-activated protein (MAP)1 kinase, ERK2, plays a critical role in promoting cellular changes in response to both mitogenic and non-mitogenic stimuli. ERK2 is the multifunctional kinase in a kinase cascade that is activated by various stimuli acting through tyrosine kinase receptors, G protein-coupled receptors, and others (1, 2). Activation of this cascade results in the dual phosphorylation of ERK2 and the consequent phosphorylation of target kinases, transcription factors, and other proteins throughout the cell. The effectors of ERK2 are localized in both the cytoplasm and the nucleus, making its subcellular localization important for its ability to induce cellular changes (3, 4).

Regulation of ERK2 localization has been characterized mostly by overexpression and/or by using antibodies to N- and C-terminal epitopes (5, 6); these epitopes are not readily accessible in ERK2 that is associated with microtubules, which constitutes a large portion of the cytoplastic protein (7). ERK2 and its upstream activator the MAP/ERK kinase MEK1 (also known as MKK1) interact stably through multiple sites with an affinity in the micromolar range (8–14). Overexpressed ERK2 accumulates in the nucleus in a stimulus-independent manner. This suggests that cytosolic binding is important in determining the distribution of ERK2 in cells. Coexpression with MEK1 shifts the distribution in favor of the cytoplasm (14). From these studies, it has been suggested that inactive ERK2 is anchored in the cytoplasm by MEK1, and that once active, ERK2 loses affinity for MEK1, moves through the nuclear pore complex (NPC), and accumulates in the nucleus (15). Overexpression studies have shown that ERK2 export can be facilitated by MEK1, which has a nuclear export sequence (NES) and requires the NES-binding protein CRM1 for movement out of the nucleus (16, 17). Nuclear entry of ERK2 has been described recently as a facilitated mechanism in which ERK2 moves through the NPC by binding directly to nucleoporins (18, 19). This process, which has been described for at least two other signaling proteins (20, 21), does not rely on the classical, karyopherin-mediated import mechanism. Thus, regulatory mechanisms governing the localization of ERK2 appear to differ significantly from those for many other proteins displaying regulated nuclear import.

Because ERK2 responds to a wide range of stimuli and controls numerous effectors throughout the cell, a number of mechanisms most likely exist to regulate its subcellular localization and thereby maintain fidelity between stimulus and stimulus-specific phenotypic change. Recently, the small anti-apoptotic protein, PEA-15, has been demonstrated to bind to ERK2 and localize it to the cytoplasm (22). PEA-15 was originally characterized in astrocytes, where it became highly phosphorylated in response to phorbol ester treatment (23). Neuronal cells express relatively high amounts of PEA-15; nevertheless, it is detected in most cultured cell lines. Astrocytes from PEA-15 knockout mice display a heightened sensitivity to tumor necrosis factor-α-induced apoptosis, indicating a protective effect of PEA-15 in the central nervous system (24).
It has also been shown to bind to the TRAIL receptor to inhibit apoptotic signaling (25). Various cancer cell lines also express relatively high amounts of PEA-15, and an increase in its mRNA is correlated with neoplastic changes in mouse mammary gland (26). Consistent with its anti-apoptotic phenotypes, PEA-15 contains a death effector domain, which is required for PEA-15 to reverse Ras-mediated suppression of integrin activation (27). PEA-15, when overexpressed, localizes ERK2 to the cytoplasm, even in the presence of stimuli that activate ERK2 (22). PEA-15 has an NES and accumulates in the nucleus in cells treated with leptomycin B, an inhibitor of CRM1-dependent nuclear export. These data suggested that PEA-15 may influence the localization of ERK2 in a similar manner to that proposed for MEK1, by binding to ERK2 and mediating its nuclear export. Thus, these studies describe one potential mechanism for the regulation of ERK2 subcellular localization by non-catalytic proteins, which may contribute to targeting ERK2 to specific cellular compartments and substrates.

To understand better the mechanism by which PEA-15 regulates ERK2 subcellular localization, we examined the behavior of ERK2 and PEA-15 using a permeabilized cell reconstitution assay, and we found that PEA-15 prevents ERK2 nuclear entry. Furthermore, PEA-15 prevents ERK2 from binding to nucleoporins in vivo, indicating it may assist in localizing ERK2 to cytoplasmic sites of action by inhibiting its movement into the nucleus.

**MATERIALS AND METHODS**

**Constructs and Recombinant Proteins**—The cDNA encoding rat ERK2 was subcloned into pSRSETBHis6-green fluorescent protein (GFP) by using KpnI and HindIII restriction sites. Mutants were generated using the QuikChange kit (Stratagene). The cDNA encoding hamster PEA-15 was a gift from Mark Ginsberg and Joe Ramos (Scirpis Research Institute and Rutgers). pGAD-ERK2 was cloned, and two-hybrid vectors encoding LexA-PEA-15 or kinase-dead MEK1 were generated by PCR as described previously (11). The constructs encoded the proteins fused to the C terminus of the LexA DNA-binding domain.

**Yeast Two-hybrid Experiments**—Pairwise interaction tests were carried out in the yeast strain L40 co-transformed with pGAD-ERK2, or the indicated ERK2 mutants, and either the empty pVJL1 (LexA) vector or pVJL1-based constructs encoding LexA fusions with sequences from PEA-15, MEK1, or MK1 as described (11). The ERK2 mutants were originally identified and described in a study of MEK1-ERK2 interactions or in an earlier study of ERK2 nuclear localization (11, 28). Co-transformants were selected, and protein-protein interactions were tested by examination of growth on co-transformant plates on medium lacking His, Leu, and Trp. The expression of all LexA and GAD fusion proteins in yeast cells was confirmed by immunoblotting with antibodies specific for either LexA or ERK1/2 (11).

**Protein Purification**—GFP-ERK2 and GFP-ERK2 mutants were prepared as described (29). GST-ERK2 was purified as described (30). PEA-15 and PEA-15 D74A each containing a His6 tag were purified on Ni2+–nitrilotriacetic acid-agarose (Qiagen) and MonoQ. Elution from the latter was performed with a step salt gradient. Karyopherin-1, karyopherin-2, Ran, p10, and glutathione S-transferase (GST)-NUP153c were purified as described (31–33). Rhodamine-labeled bovine serum albumin (BSA) was coupled to a peptide containing the nuclear localization sequence (NLS) of the simian virus 40 T antigen to make a biotinylated substrate NLS-BSA (32).

**Import Assays**—Import assays were performed as described by using REFS2 cells grown in medium supplemented with 10% fetal bovine serum and 1% glutamine (31). Cells plated on coverslips 24 h before permeabilization were washed once with import buffer (20 mM Hepes-serum and 1% glutamine (31)). Cells plated on coverslips 24 h before import, were incubated with 0.5 μg/ml phenylenediamine in 90% phosphate-buffered saline plus 10% glycerol. GFP and TRITC were visualized by fluorescence microscopy with a Zeiss Axiocam microscope equipped with a Hamamatsu Orca II camera. Pictures for individual experiments were taken using identical exposures. Uptake was quantified by using Improvision OPEN LAB software.

**Binding Experiments**—The method described by Werner and co-workers (34) was used to measure binding of purified GST-ERK2 wild type and mutants to His6-PEA-15, except that bovine serum albumin (3 mg/ml) was included in the incubations, and interactions were detected by immunoblotting with an anti-His6 monoclonal antibody (Clontech). To measure ERK2 binding to Nup153c, GST-Nup153c (2 μM) was incubated for 30 min with glutathione-agarose equilibrated in import buffer at 4°C. His6-ERK2 (200 μM) was added with or without the indicated ratios of PEA-15 and mixed end over end at 4°C for 2 h. Bovine serum albumin (6 mg/ml) was present in the binding reactions. Binding was washed four times with 1 μM Nup153c, 0.1% Triton X-100 in import buffer and analyzed on a 10% polyacrylamide gel in SDS. Proteins were transferred to nitrocellulose and immunoblotted with anti-serum Y691 for 1 h at 1:1000 dilution (35).

**Kinase Assays**—Kinase assays were performed as described previously. ERK2 (0.8 μM) was incubated with 120 μM MEK1R4F (38), 10 μM ATP, and increasing amounts of PEA-15. Reactions were incubated for 60 min at 30°C. Proteins were resolved on gels as above and subjected to autoradiography. Counts/min incorporated into ERK2 were measured by liquid scintillation counting.

**RESULTS**

To analyze the possible site of interaction of PEA-15 with ERK2, we first demonstrated that we could measure their binding using a directed two-hybrid test (Fig. 1A and Table I). We then assessed the binding of PEA-15 to the following ERK2 mutants that are defective in interactions with other proteins, some of which were identified from an ERK2 mutant library (11): 1) ERK2 D316A, D319A (DDAA), which lacks two aspartic acid residues required for binding to proteins with basic/hydrophobic docking (D) motifs including MEK1, the substrates p90 Rsk, MNK1, Elk1, and c-Fos, and the MAP kinase phosphatase MKP-3 (11, 14, 37–39); 2) ERK2 lacking the MAP kinase insert (residues 241–272; ERK2Δ241–272), which is not activated by MEK1 or MEK2, and 3) four other ERK2 point mutants, Y261N/L, L235P, N236I, and G243R, that are variably defective in binding to MEK1 (Fig. 1B). PEA-15 bound to ERK2 (DDAA) as well as it bound to wild type ERK2, indicating that PEA-15 does not bind to ERK2 through a basic/hydrophobic D domain. However, PEA-15 did not interact with ERK2Δ241–272, although this mutant retained binding to MNK1 and MKP-3 (11). Loss of a two-hybrid interaction suggests a substantial decrease in the affinity of their association. The point mutants that displayed minimal binding to PEA-15 were all located in or near the MAP kinase insert and included Y261N/L, which is the most defective in binding to MEK1 based on its inability to be activated in cells (Table I) (11). No functions of the MAP kinase insert are known other than for productive binding of ERK2 to MEK1. The binding data here indicate that a second function of the MAP kinase insert is to interact with PEA-15.

To validate the results of the two-hybrid tests, the binding of the two key GST-ERK2 mutants to PEA-15 was assessed using glutathione-agarose to harvest bound proteins (Fig. 1C). As expected, ERK2 (DDAA) associated with PEA-15 in the pull-down assays, but ERK2Δ241–272 bound poorly to GST-PEA-15, consistent with the two-hybrid findings.

As indicated above, the region of ERK2 that was necessary for the interaction with PEA-15 overlapped that identified previously (11) as required for the capacity of MEK1 to activate ERK2 in cells. This observation suggested that PEA-15 should also interfere with the interaction of ERK2 with MEK1. To determine whether this was the case, we included PEA-15 in kinase assays with ERK2 and a constitutively active form of MEK1, MEK1 R4F. Under these conditions, we could assess competition as a loss of ERK2 phosphorylation and activation (Fig. 2). A 10-fold molar excess of PEA-15 in the kinase assays was sufficient to reduce ERK2 activation by MEK1 to less than
20% of that in its absence, consistent with the idea that PEA-15 interferes with a productive interaction of MEK1 with ERK2. Overexpression studies demonstrated that PEA-15 can induce accumulation of ERK2 in the cytoplasm. This accumulation was attributed to regulation of ERK2 export by PEA-15, which has an NES (22). To determine the effects of PEA-15 on ERK2 localization, we included PEA-15 in an import reconstitution assay with GFP-ERK2. In vitro import assays are performed by permeabilizing the plasma membrane with digitonin. Digitonin permeabilization results in the loss of soluble proteins including proteins required for conventional import and export processes, notably the small G protein Ran, but leaves the nucleus and NPC intact. We have shown previously that the control import substrate, NLS-BSA, is not imported in the absence of added transport factors and energy in this system (19). In contrast, GFP-ERK2 enters the nucleus in the absence of added soluble proteins and energy in a manner facilitated by interactions with the FXF-rich nucleoporins, which are major components of the nuclear pore (18, 19). Protein outside the nucleus that is not tightly bound will wash away prior to visualization. Entry of GFP-ERK2 into the nucleus is inhibited by the lectin wheat germ agglutinin, which binds to N-acetylglucosamine residues on nucleoporins and occludes the NPC (19). If we included PEA-15 in these assays, we found a significant inhibition of GFP-ERK2 entry at 15 min. The inhibition of entry was dependent on the PEA-15 concentration. Reduced entry of GFP-ERK2 was clearly detectable if a 7.5–10-fold molar excess of PEA-15 was included in the assay (Fig. 3A). We also performed additional assays including recombinant transport factors, βH9251-karyopherin, βH9252-karyopherin, p10, Ran, and an energy reconstitution system (19), to determine whether nuclear accumulation of GFP-ERK2 was influenced by PEA-15 in the presence of transport factors. Under these conditions, nuclear accumulation of GFP-ERK2 was still inhibited by PEA-15. On the other hand, PEA-15 had no effect on the entry of the control import substrate NLS-BSA (Fig. 3C), indicating that PEA-15 does not generally disrupt conventional nuclear import. Because an energy-regenerating system, Ran, and CRM1 were not required in the permeabilized cell system, inhibition of the nuclear accumulation of GFP-ERK2 is most likely not due to CRM1-mediated export of a PEA-15-GFP-ERK2 complex. Our findings with PEA-15 are consistent with work by Nishida and co-workers (18) who reported previously that MEK1 can prevent the nuclear entry of GFP-ERK2 in similar reconstitution assays. Thus, we conclude that the ability of PEA-15 to prevent nuclear accumulation of ERK2 in this assay must be due to its sequestration outside the nucleus.

To determine whether cytoplasmic retention of GFP-ERK2 caused by PEA-15 required a direct interaction with ERK2, we tested the effect of PEA-15 on nuclear import of ERK2 with mutations noted above. GFP-ERK2 DDAA entered the nucleus like wild type ERK2, and its entry was reduced by PEA-15 (Fig. 3B).

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**Table I**

The ability of ERK2 wild type and mutants to bind to PEA-15 by yeast two-hybrid analysis

| Mutation | MEK1-K97M | PEA-15 | MNK1 |
|----------|-----------|--------|------|
| WT       | +         | +      | +    |
| L235P    | −         |        | +    |
| L235P,N236I | −     | −      | +    |
| G243R    | −         | −      | +    |
| Y261C,Y261N | −     | −      | +    |

Plus signs indicate an interaction; minus signs indicate no detectable interaction.
This result was consistent with the binding data that showed that mutation of the two aspartic acid residues in ERK2 DDAA did not reduce PEA-15 binding (Fig. 1, A and C). GFP-ERK2Δ241–272 was also capable of nuclear entry, but in contrast, its entry was much less sensitive to the presence of PEA-15; reduced entry was only observed at the highest PEA-15 concentration (Fig. 4, A and B). In the presence of PEA-15 no entry of wild type ERK2 was observed even at 1 min, the shortest time of observation, nor was it observed at any time over the time course up to 30 min (Fig. 4C). ERK2Δ241–272 displayed a similar accumulation over time even in the presence of PEA-15 (Fig. 4D). No inhibition of nuclear entry was observed with ERK2 Y261N (Fig. 5A and Table II), which was the point mutant most impaired in binding to MEK1 and PEA-15 by the two-hybrid test (11). In addition, a mutant in the death effector domain of PEA-15, D74A, that reportedly fails to bind to ERK2 was also tested in the reconstitution assay. In the presence of PEA-15 D74A, nuclear entry of GFP-ERK2 was detected; the extent was intermediate between that observed in the absence and presence of wild type PEA-15 (Fig. 5B). This result is consistent with the decreased ERK2 binding of this mutant. Thus, we conclude that binding of PEA-15 to ERK2 induces a cytoplasmic localization of ERK2 in this assay by directly preventing its nuclear entry.

We showed previously (28) using microinjection of recombinant proteins into the cytoplasm of REF52 cells that ERK2 K52R, a kinase-dead mutant, and ERK2 T183A, lacking one of two essential phosphorylation sites in the activation loop, could also accumulate in the nuclei of stimulated cells. Thus, we wished to test the ability of these ERK2 mutants to be imported into the nucleus in the import reconstitution system and the effect of PEA-15 on their entry. We found that GFP-ERK2 K52R and T183A both accumulated in the nucleus in a manner similar to wild type ERK2 (Fig. 6). In both cases, PEA-15 blocked their nuclear accumulation. We had also shown previously that the mutant ERK2 L4A H176E, which was unable to form dimers due to mutations in the dimer interface, displayed a reduced ability to accumulate in the nucleus of stimulated cells (28). Interestingly, this dimer mutant localized to the nucleus in the import reconstitution assay in a manner similar to wild type ERK2 (Fig. 6). This suggests that a process distinct from facilitated entry causes the abnormal distribution of this mutant in cells. As with wild type ERK2, PEA-15 was able to prevent the nuclear entry of the dimer mutant. The dimer interface of ERK2 consists of residues in the N-terminal domain and the activation loop, all distant from the MAP kinase insert. These results are consistent with the earlier assessment that the MAP kinase insert is the major site of interaction of PEA-15 with ERK2.

Current evidence suggests that ERK2 binding to nucleoporins is required for its rapid nuclear entry in the import assay; therefore, we wished to determine the effect of PEA-15 on the ERK2-nucleoporin interaction. We and others have (18, 19) shown previously that ERK2 binds to nucleoporins (NUP 153c and NUP214) in GST pull-down assays. Both of these porins contain numerous FXF repeats, which are found in a number of ERK2 substrates (40). Therefore, we tested the ability of ERK2 to bind to the nucleoporin NUP153c in the presence of increasing concentrations of PEA-15 (Fig. 7). We found that an excess of PEA-15, similar to the amounts used in the import assay, significantly decreased ERK2 binding to NUP153c. These results are consistent with the earlier assessment that the MAP kinase insert is the major site of interaction of PEA-15 with ERK2.

We showed previously (28) using microinjection of recombinant proteins into the cytoplasm of REF52 cells that ERK2 K52R, a kinase-dead mutant, and ERK2 T183A, lacking one of two essential phosphorylation sites in the activation loop, could also accumulate in the nuclei of stimulated cells. Thus, we wished to test the ability of these ERK2 mutants to be imported into the nucleus in the import reconstitution system and the effect of PEA-15 on their entry. We found that GFP-ERK2 K52R and T183A both accumulated in the nucleus in a manner similar to wild type ERK2 (Fig. 6). In both cases, PEA-15 blocked their nuclear accumulation. We had also shown previously that the mutant ERK2 L4A H176E, which was unable to form dimers due to mutations in the dimer interface, displayed a reduced ability to accumulate in the nucleus of stimulated cells (28). Interestingly, this dimer mutant localized to the nucleus in the import reconstitution assay in a manner similar to wild type ERK2 (Fig. 6). This suggests that a process distinct from facilitated entry causes the abnormal distribution of this mutant in cells. As with wild type ERK2, PEA-15 was able to prevent the nuclear entry of the dimer mutant. The dimer interface of ERK2 consists of residues in the N-terminal domain and the activation loop, all distant from the MAP kinase insert. These results are consistent with the earlier assessment that the MAP kinase insert is the major site of interaction of PEA-15 with ERK2.

Fig. 3. PEA-15 inhibits nuclear entry of GFP-ERK2. A, GFP-ERK2 (0.8 μM, 50 μg/ml) was added alone or with PEA-15 (0.6, 6, or 60 μM) for 15 min. Arrows indicate nuclei. One of six experiments performed in duplicate is shown. B, import assays with 0.8 μM GFP-ERK2 were performed alone and with 80 μM PEA-15 with or without transport factors and an energy-regenerating system for 15 min. Arrows indicate nuclei. One of two experiments is shown. C, as in A but NLS-BSA (5.0 μg/ml) was added alone or with 80 μM PEA-15 for 15 min. One of two experiments is shown.
Results suggest that PEA-15 interferes with the association of ERK2 with nucleoporins. In view of the two-hybrid data and the assays here, we estimate that the $K_d$ for ERK2-PEA-15 binding is near $1/\mu \text{M}$, consistent with the concentration required for inhibitory effects in the import assays. PEA-15 itself, does not appear to bind to nucleoporins, consistent with import assay findings that PEA-15 does not inhibit import of NLS-BSA, which uses the classical nuclear import system (Fig. 3C); the karyopherins themselves must bind to nucleoporins to escort their import cargo into the nucleus.

**FIG. 4.** Nuclear accumulation of GFP-ERK2 and mutants in the presence of PEA-15. A, import assays with GFP-ERK2 (one of five experiments), GFP-ERK2 D316A, D319A (one of two experiments), and GFP-ERK2Δ241–272 (one of five experiments) with 60 μM PEA-15 for 15 min. WT, wild type. B, quantitation of nuclear staining following import assays with GFP-ERK2 and GFP-ERK2 DDAA (one of two experiments), or GFP-ERK2Δ241–272 (one of three experiments) alone (white bars) or with 0.6, 6, or 60 μM PEA-15 (black bars) for 15 min. C, quantitation of nuclear staining following a time course import assay with GFP-ERK2 alone (white bars) and 60 μM PEA-15 (black bars). One of eight experiments is shown. D, quantitation of nuclear fluorescence in time course import assays with GFP-ERK2Δ241–272 alone (white bars) or with 60 μM PEA-15 (black bars). One of eight experiments is shown.

**FIG. 5.** ERK2 and PEA-15 mutants in the import assay. A, import assay as in Fig. 4 with GFP-ERK2 and GFP-ERK2 Y261N with and without 60 μM PEA-15 for 15 min. Arrows indicate nuclei. WT, wild type. One of four experiments is shown. B, import assay as above with GFP-ERK2 and 60 μM PEA-15 D74A for 15 min. One of four experiments is shown.

**TABLE II**

Effect of PEA-15 on import of ERK2 and mutants in the import assay

| Mutation                  | PEA-15-sensitive |
|---------------------------|------------------|
| WT                        | +                |
| D316A,D319A               | +                |
| H176E,L333A,L336A,L341A,L344A | +                 |
| K52R                      | +                |
| T183A                     | +                |
| Δ241–272                  | −                |
| Y261N                     | −                |

Results suggest that PEA-15 interferes with the association of ERK2 with nucleoporins. In view of the two-hybrid data and the assays here, we estimate that the $K_d$ for ERK2-PEA-15 binding is near $1/\mu \text{M}$, consistent with the concentration required for inhibitory effects in the import assays. PEA-15 itself, does not appear to bind to nucleoporins, consistent with import assay findings that PEA-15 does not inhibit import of NLS-BSA, which uses the classical nuclear import system (Fig. 3C); the karyopherins themselves must bind to nucleoporins to escort their import cargo into the nucleus.
Once in the presence of PEA-15, performed three times in the absence and without 60 μM PEA-15 for 15 min. Arrows indicate nuclei. WT, wild type. Note binding of ERK2 T183A to cytoskeletal elements. Import experiments were performed three times in the absence and once in the presence of PEA-15.

**Fig. 6.** Nuclear accumulation of GFP-ERK2 mutants in the presence of PEA-15. Import assays as in Fig. 4 with GFP-ERK2 and the mutants K52R, T183A, H176E/L4A, and ΔΔ41–272 with and without 60 μM PEA-15 for 15 min. Arrows indicate nuclei. WT, wild type.

**Fig. 7.** PEA-15 inhibits ERK2 binding to NUP153c. GST pull-down assay using 2 μM GST or GST-NUP153c and 0.2 μM ERK2 alone or with 0.2, 2, or 20 μM PEA-15. One of five experiments is shown.

**DISCUSSION**

ERK2 phosphorylates proteins in multiple subcellular localizations. Movement of ERK2 within the cell is essential for targeting ERK2 to substrates in different compartments to produce discrete, ligand-specific phenotypes. The growth factor-dependent movement of ERK2 from the cytoplasm to the nucleus has been amply documented in PC12, 3T3, REF52, HeLa, CCL39, Chinese hamster ovary, and other cell types (3, 7, 41–44). Unlike most other proteins displaying this behavior, ERK2 apparently does not enter the nucleus by the classical import mechanism; instead, ERK2 is capable of moving bidirectionally across the NPC independently of soluble carrier proteins by directly binding to FXF motifs in nucleoporins (18, 19).

ERK2 also activates substrates in the cytoplasm. In some cases a fraction of it may be tethered in the cytoplasm until a ligand-dependent release; perhaps its behavior is like that of β-catenin, which is retained in the cytoplasm through interaction with cytoskeletal elements until signaled for release to carry out its nuclear transcriptional regulatory function (45). β-Catenin, like ERK2, enters the nucleus by binding to nucleoporins (20). The identification of proteins that mediate regulation of ERK2 movement and anchoring at the membrane, in the cytoplasm, and in the nucleus, as well as their mechanisms of action will be important in understanding how localization of ERK2 is controlled in a stimulus-dependent manner.

Several proteins have been implicated in preventing the nuclear accumulation of ERK2. In addition to MEK1, these include calponin, a protein highly expressed in smooth muscle, and PEA-15 (15, 22, 46). Overexpression of PEA-15 is sufficient to maintain a cytoplasmic localization of ERK2, even under conditions that activate it and would normally result in its nuclear accumulation. PEA-15 reportedly binds to ERK2 in both inactive and active forms (22). Because PEA-15 itself has an NES, it has been proposed that export of PEA-15 from the nucleus via CRM1 also results in export of ERK2 complexed to it. However, no direct evidence for this mechanism has been reported. Here we have examined the mechanism by which PEA-15 alters the localization of ERK2. Our results do not rule out a contribution to the PEA-15-dependent localization of ERK2 through accelerated export. Nevertheless, we demonstrate the unexpected result that PEA-15 has the capacity to sequester ERK2 outside of the nucleus, in a manner independent of nuclear export. We found no significant nuclear uptake of PEA-15 in the import assay in the absence or presence of energy and cytosolic transport factors (data not shown), suggesting that it may enter the nucleus at a relatively slow rate. If so, the effect of PEA-15 to block ERK2 import may be more significant than the action on ERK2 export. Under the conditions of the import reconstitution assay, NES-mediated export is not observed without the addition of CRM1 and Ran. Together with the kinetics of entry in the presence and absence of PEA-15, these findings indicate that the lack of nuclear ERK2 signal in our experiments can only be attributed to failed nuclear entry.

By using a random mutagenesis strategy, we found that MEK1, the upstream activator of ERK2, binds to the ERK2 MAP kinase insert (11); the two-hybrid and in vitro binding results here indicate that this region of ERK2 is also important for binding to PEA-15, supporting a report from Werner and co-workers (34). MEK1 contacts a significant portion of the ERK2 surface (9). Among well defined binding contacts, the MAP kinase insert is the site that is required for productive interaction between ERK2 and MEK1 that mediates ERK2 phosphorylation by MEK1 (11). Because PEA-15 requires the MAP kinase insert to bind tightly to ERK2, it is not surprising that PEA-15 reduces the in vitro activation of ERK2 by MEK1. This finding seems to conflict with data indicating that overexpression of PEA-15 increases ERK2 activation, both duration and amplitude, in Chinese hamster ovary cells (22). Perhaps in cells, inhibition of ERK2 activation by overexpressed PEA-15 is not observed either because the relocation of ERK2 caused by PEA-15 prevents ERK2 inactivation by phosphatases or the reduced interaction of ERK2 with MEK1 is compensated for by other proteins such as scaffolds that promote their association.

PEA-15 apparently anchors ERK2 in the cytoplasm. Previous reports (22) have demonstrated that this is independent of ERK2 activation state. In agreement with this, here we find that a kinase-dead mutant and a phosphorylation site mutant of ERK2 are sensitive to PEA-15-mediated inhibition of nuclear import, although they enter the nucleus like wild type ERK2 in the absence of PEA-15. This latter finding is consistent with the lack of a requirement for ERK2 activity in the facilitated entry process. The ability of the active form to be retained at sites of action may be substantially due to its affinity for substrates at those sites. For example, there is evidence that ERK2 is anchored in the nucleus in stimulated cells, perhaps through interactions with the mitotic apparatus or transcriptional regulators (47–49). Based on results of an earlier study, we suggested that dimerization of phosphorylated ERK2 might
cause retention of ERK2 in the nucleus, perhaps due to its inability to be exported in complexes when dimeric (28). We found here that the dimer-defective mutant of ERK2 enters the nucleus like wild type ERK2. This suggests that the ability to dimerize is not required for nuclear entry and is consistent with the hypothesis that the entry of ERK2 into the nucleus is not the predominant regulated step in its stimulus-dependent nuclear accumulation.

The effect of PEA-15 on ERK2 nuclear entry depends on its direct interaction with ERK2, rather than general interference with import mechanisms, because mutations in ERK2 or PEA-15 that decrease their affinity reduce the ability of PEA-15 to inhibit ERK2 import. Our work suggests that at least one mechanism of action of PEA-15 is through the inhibition of the interaction of ERK2 with nucleoporins in the NPC, thereby preventing ERK2 nuclear entry. Interfering with nucleoporin binding of ERK2 by other means has been shown to prevent entry of ERK2 into the nucleus (19).

The regions on ERK2 that bind FXF motifs of nucleoporins or other FXF-bearing proteins, such as Fox or Elk, have not been unequivocally identified. The fact that ERK2, which lacks the MAP kinase insert, enters the nucleus in the import assay indicates that the insert itself is not an essential element of the FXF-binding site. However, the interference of PEA-15 with ERK2 binding to NUP153c and the ability of PEA-15 to prevent the nuclear entry of ERK2 both support the hypothesis that PEA-15 directly interferes with the association of ERK2 with nucleoporins. Alternatively, through its contact with the MAP kinase insert, PEA-15 may induce a conformational change elsewhere on ERK2 that disorders the FXF-binding site. A further hint that binding of a protein to the MAP kinase insert might block nuclear entry comes from the observation that ERK2Δ241–272 displays a subtle defect in nuclear uptake, most obvious as slower kinetics of entry. Finally, in support of this argument, MEK1, which binds to the MAP kinase insert of ERK2, is well documented to localize ERK2 to the cytoplasm. This may also be due in part to nuclear export of an ERK2-MEK1 complex, but Nishida and co-workers (18) have also shown that MEK1 retains ERK2 in the cytoplasm in import reconstitution assays comparable with those used here. Thus, the MAP kinase insert, although not essential for FXF binding, may be located near or allosterically impact the FXF-binding motif. The weak interaction of ERK2 with FXF motifs has, thus far, prevented us from using the two-hybrid approach to identify the binding site. The identification of FXF binding regions on ERK2 is under intense study in a number of laboratories.

Expression of PEA-15 is low in most cell lines, except those of neuronal origin, indicating that only a fraction of ERK2, which is relatively abundant, is interacting and being sequestered by PEA-15. This small fraction of ERK2 may be sufficient to prevent apoptosis in certain cellular contexts. PEA-15, or other non-catalytic proteins, may be important for retaining ERK2 in the cytoplasm to perform specific functions. One of these may be its role in survival; PEA-15 is an anti-apoptotic protein, and a recent study (50) has shown that phosphorylation of caspase 9 by ERK2 prevents the activation of the apoptotic cascade. These cytoplasmic functions have often been neglected in comparison with the role of ERK2 in phosphorylating nuclear substrates such as transcription factors, but nevertheless are essential to ERK2 function. Furthermore, the ability of small non-catalytic proteins to integrate multiple signals may also be crucial for ensuring proper cellular responses to multiple stimuli.

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45. Moon, R. T., Bowerman, B., Boutros, M., and Perrimon, N. (2002) Science 296, 1644–1646
46. Menice, C. B., Hulvershorn, J., Adam, L. P., Wang, C. A., and Morgan, K. G. (1997) J. Biol. Chem. 272, 25157–25161
47. Lenormand, P., Brundello, J. M., Brunet, A., and Pouyssegur, J. (1998) J. Cell Biol. 142, 625–633
48. Shapiro, P. S., Vaisberg, E., Hunt, A. J., Tolwinski, N. S., Whalen, A. M., McIntosh, J. R., and Ahn, N. G. (1998) J. Cell Biol. 142, 1533–1545
49. Shapiro, P. S., Whalen, A. M., Tolwinski, N. S., Freelich-Ammon, S. J., Garcia, M., Osheroff, N., and Ahn, N. G. (1999) Mol. Cell. Biol. 19, 3551–3560
50. Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., and Clarke, P. R. (2003) Nat. Cell Biol. 5, 647–654
51. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H., and Goldsmith, E. (1997) Cell 90, 859–869