Mutations in ERK2 Binding Sites Affect Nuclear Entry*

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The MAPK ERK2 can enter and exit the nucleus by an energy-independent process that is facilitated by direct interactions with nuclear pore proteins. Several studies also suggest that the localization of ERK2 can be influenced by carrier proteins. Using import reconstitution assays, we examined a group of ERK2 mutants defective in known protein interactions to determine structural properties of ERK2 that contribute to its nuclear entry. ERK2 mutants defective in binding to substrates near the active site or to basic/hydrophobic docking (D) motifs were imported normally. Several ERK2 mutants defective in interactions with FXF motifs displayed slowed rates of nuclear import. The import-impaired mutants also showed reduced binding to a recombinant C-terminal fragment of nucleoporin 153 that is rich in FXF motifs. Despite the deficit revealed in some mutants via reconstitution assays, all but one of the ERK2 mutants accumulated in nuclei of stimulated cells in a manner comparable with the wild type protein; the mutant most defective in import remained in the cytoplasm. These results further support the idea that direct interactions with nucleoporins are involved in ERK2 nuclear entry and that multiple events contribute to the ligand-dependent relocalization of these protein kinases.

The nuclear localization of the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 has been intensely explored because their regulated nuclear accumulation has been linked to their ability to change cell programs such as differentiation and proliferation (1–4). The altered localization of these kinases has also been reported in disease (5).

The first studies of ERK1/2 localization indicated that ligands and other stimuli caused their nuclear translocation and that phosphorylation, which activates the protein kinases, is sufficient to promote their redistribution (1). An early indication that regulation of nuclear localization might be more complex came from microinjection studies with unphosphorylated ERK2 (6). Protein injected into the cytoplasm of fibroblasts rapidly entered the nucleus and then redistributed to the cytoplasm, suggesting that phosphorylation is not essential for nuclear entry but might be involved in nuclear retention.

Two laboratories used import reconstitution to show that unphosphorylated ERK2 enters the nucleus by a process distinct from the well characterized carrier-mediated mechanisms (7, 8). Nuclear uptake of ERK2 requires neither energy nor carrier proteins and has been proposed to occur by the binding of ERK2 directly to nuclear pore proteins. Two different nucleoporins (Nups), Nup153 and Nup214, were shown to bind to ERK2 in vitro. More recently a second import mechanism was found to occur with active, phosphorylated ERK2 (pERK2) but not with the unphosphorylated protein; this second import process requires energy and cytosolic factors (9).

Studies of ERK2 export using reconstitution in permeabilized cells concluded that, independent of its phosphorylation state, the export of ERK2 can occur by at least two processes. One process is carrier- and energy-independent and is consistent with the capacity of the Nup interaction to mediate bidirectional movement of ERK2, both into and out of the nucleus. Substantiation for the facilitated import and export mechanism came from studies using fluorescence recovery after photobleaching, which supported the conclusion that the nuclear entry and exit of ERK2 was rapid and did not require energy (10). The second export process is active and is dependent on the export carrier CRM1 (9). A role for CRM1 had already been deduced from the ability of the CRM1 inhibitor leptomycin B to increase the nuclear concentration of ERK1/2 (11).

A variety of studies have suggested the importance of ERK1/2-binding proteins in determining their subcellular localization. The existence of a labile nuclear anchoring protein was inferred from a kinetic analysis of ERK1/2 activation and nuclear retention (12). Mxi2, an unusual splice variant of p38 MAPK, promotes nuclear translocation and retention of ERK1/2 (13, 14). MEK1/2, the upstream activators of ERK1/2, have been implicated in their cytoplasmic retention (11, 15). Overexpressed ERK2 accumulates in the nucleus even when not phosphorylated (8, 15); when overexpressed, ERK2 is assumed to exceed MEK1/2 in concentration, resulting in an excess of free ERK2 to accumulate in the nucleus.
MATERIALS AND METHODS

Constructs and Recombinant Proteins—His6-tagged GFP-ERK2 (rat), pERK2, and MEK1-4F were expressed and purified as described (9, 22). The following His6-GFP-ERK2 mutants were described (23): K52R, T183A, L333A/L336A/L341A/L344A/H176E (referred to as L4A/H176E), D316A/D319A, Y261N, and Δ241–272 (deletion of the MAPK insert). Rhodamine-labeled bovine serum albumin (BSA) coupled to a synthetic nuclear localization sequence (NLS) was described previously (23). Additional ERK2 mutants were generated by site-directed mutagenesis using the QuikChange kit (Stratagene).

Cell Culture—HeLa, human embryonic kidney 293, and BJ cells (human foreskin fibroblasts immortalized with h-TERT) (24) were grown on coverslips for 24 h in Dulbecco’s modified Eagle’s medium at 37 °C in 10% CO2 supplemented with 10% fetal bovine serum and 1% L-glutamine and, in early experiments, 100 units/ml penicillin/streptomycin. BJ cells were incubated in serum-free medium for 2 h prior to use in import assays with pERK2.

Transfection—Human embryonic kidney 293 and HeLa cells were transfected with Myc-ERK2 constructs using FuGENE 6 following the manufacturer’s protocol (Roche Applied Science). After 48–72 h, cells were lysed in Triton X-100 lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5 mM sodium orthovanadate, 20 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Samples were resolved by SDS-PAGE and analyzed by immunoblotting with appropriate antibodies after transferring to nitrocellulose membranes.

Anti-Myc was from the National Cell Culture Center.

Import Assays—Import and export assays were as described (23, 25). Cells were washed once in transport buffer (TB) (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′′,N′′′-tetraacetic acid (EGTA) and 2 mM dithiothreitol) and permeabilized for 5 min with 70 μg/ml digitonin. Import assays were for 15 min or as indicated in 40 μl of TB with the following: 10 mg/ml BSA and either 0.8 μM recombinant ERK2 substrate (wild type or mutant GFP-ERK2 or pERK2) or 0.14 μM tetramethylrhodamine B isothiocyanate (TRITC)-NLS-BSA. In the indicated experiments dialyzed HeLa cytosol (2.5 mg/ml) was included as a source of transport factors along with an energy-regenerating system containing 1 mM ATP, 1 mM GTP, 5 mM phosphocreatine, and 20 units/ml creatine phosphokinase. After addition of 0.25 ml of TB, cells were either fixed or used for export assays as described next. To examine export, preloaded cells were placed in 40 μl of TB with 10 mg/ml BSA for 30 min or as indicated. The reaction was terminated with 0.25 ml of TB. Cells were fixed for 10 min in 3% paraformaldehyde; coverslips were mounted with polypl view.

pERK2 was detected by immunofluorescence in fixed cells that had been permeabilized in 0.5% Triton X-100 for 10 min. Cells were blocked in 1% Triton-buffered saline (TBS), 0.1% Tween 20, and 10 mg/ml BSA for 30 min at room temperature and incubated with the pERK1/2 antibody (Sigma) at 1:300 for 16 h at 4 °C; the secondary antibody, Alexa-546 anti-mouse (Molecular Probes), was used at 1:3000 for 1 h. Washes between incubations were performed three times with TBS and 0.05% Tween 20 for 10 min.

Fluorescence Microscopy—Fluorophores were visualized by fluorescence microscopy using a Zeiss Axioskop 2-plus microscope and a Hamamatsu digital CCD camera (C4742–95). Exposures for all conditions within an experiment were kept the same; fluorescence intensity was quantified using Slidebook 4.1 software (Intelligent Imaging Innovations, Inc.).
**TABLE 1**

| Import of ERK2 and mutants |
|---------------------------|
| **Summary of in vitro import assays. Values shown are import in 20 min relative to wild type ERK2 or pERK2 rounded to the nearest 5% based on analyzing 100 cells for each condition.** |
| **Mutant** | **ERK2 - E** | **pERK2 - E** | **pERK2 + E** | **Wild type** |
| ERK2 | 100 | 100 | 100 | Wild type |
| Y261N or A | 35 | 35 | 75 | FXF binding |
| I196/M197A | 45 | 75 | 80 | FXF binding |
| N199/S200A | 55 | 100 | 100 | FXF binding |
| Y231/232A | 100 | 45 | 75 | FXF binding |
| Δ241–272 | 25 | – | – | -MAPK insert |
| D316/319A | 90 | 95 | 100 | D motif binding |
| E320K | 100 | 100 | 90 | D motif binding |
| W190A | 100 | 95 | 100 | Substrate site |
| P224G | 95 | 80 | Substrate site |
| P227G | 95 | 90 | Substrate site |
| K25R | 100 | 100 | Inactive |
| L_A/H176E | 100 | 100 | Monomeric |

**RESULTS**

**Known Binding Sites on ERK2**—To explore the role of ERK2 interactions in its nuclear entry, we have examined the behavior of ERK2 mutants, unphosphorylated and phosphorylated, using reconstitution assays in permeabilized BJ cells. BJ cells are human foreskin fibroblasts immortalized with human telomerase (24). Two regions of ERK2 have been identified as docking sites for substrates and regulators that lie outside of the protein kinase active site. One region includes acidic residues (common docking site) and a hydrophobic groove on the C-terminal domain; this region binds to basic/hydrophobic docking motifs in interacting proteins (27–30). The other region includes residues in a-helix G and the MAPK insert and binds substrates with FXF motifs (also called DEF motifs), as well as PEA-15 (19, 31, 32). We have characterized the behavior of the mutants in these two docking regions and nearer the active site of ERK2 (Fig. 1). These reconstitution experiments use a limited set of defined conditions and assess a pre-steady state extent of import, thereby allowing us to discern subtle differences in the import properties of ERK2 mutants. Results of import assays are summarized in Table 1.

**Import of ERK2 Mutated in the Putative Nup Binding Site**—We previously showed that PEA-15 blocks binding of ERK2 to Nups (19). Thus, we tested the idea that ERK2 mutants that displayed reduced binding to PEA-15 might also interact less well with Nups. In a screen for ERK2 mutants with reduced binding to interactors, we found that ERK2 Y261N showed reduced binding to PEA-15 (8, 19). This residue lies within the MAPK insert, residues 241–272 of ERK2 (Fig. 1). Deletion of the MAPK insert also reduced binding to PEA-15 and resulted in a defect in ERK2 import (19).
We further characterized the import of these MAPK insert mutants over a longer time course in the absence (Fig. 2A) and presence (Fig. 2C) of cytosol, to provide import factors, and an energy-regenerating system, to maintain sufficient GTP for the Ran-GTP cycle. Under these conditions, a defect in import of GFP-ERK2 Y261N relative to wild type ERK2 was also observed. GFP-ERK2-(Δ241–272) displayed a slower rate of import than wild type ERK2 or the point mutant at all times and under both conditions. Quantitation indicated that Y261N was imported better in the presence of cytosol and energy, reaching ~60% of the intensity of wild type ERK2 after 2 h (Fig. 2, B and D) (19).

Nups contain numerous FXF motifs. FXF motifs are present in a number of ERK2 substrates and bind directly to ERK2. We previously proposed that the Nup binding site was also the site for binding to FXF motifs of substrates (19). Hydrogen-deuterium exchange studies demonstrated the location of this site; a peptide from Elk-1 containing an FXF motif bound to pERK2 between the MAPK insert and α-helix G (32). An examination of the crystal structures of ERK2 and pERK2 suggested that phosphorylation of ERK2 may affect the accessibility of this hydrophobic binding site. Based on the structure, residues that might interact with the Nup FXF motif in ERK2 were predicted; some were different from the pERK2 residues contacting Elk-1. These residues and ones revealed by the hydrogen-deuterium

FIGURE 3. Import of ERK2 proteins mutated in the putative FXF binding site. A and B, import of the indicated mutants was performed for 20 min in transport buffer without added energy or cytosol. Import of rhodamine-labeled NLS-BSA with cytosol and energy for 20 min is shown as a control in panel A. Fluorescence of cells not incubated with GFP-ERK2 is shown as a control in panel B. C, quantitation of fluorescence intensity within the nuclei of experiments shown in panels A and B. Error bars are standard deviation.

FIGURE 4. Import of pERK2 and mutants. A and C, import of ERK2 and the indicated ERK2 mutants phosphorylated in vitro was performed for 20 min in transport buffer without added energy or cytosol. B and D, quantitation of fluorescence intensity within the nuclei of experiments shown in panels A and C. E, pERK2 blots of in vitro phosphorylated ERK2 and mutants. ERK2-(Δ241–272) cannot be phosphorylated by MEK1 (8). The last lane contains unphosphorylated ERK2. Error bars are standard deviation.
ERK2 Import Mutants

Nucleoporin Binding Is Decreased in the Import-defective Mutants—Because ERK2 binds to nucleoporins directly, decreased import might result from decreased nucleoporin binding. The results above suggest that some overlapping and some unique residues of ERK2 and pERK2 interact with Nups. A test to determine whether this may be the case, we tested binding of unphosphorylated (Fig. 5A) and phosphorylated (Fig. 5B) GFP-ERK2 proteins to a C-terminal fragment of Nup153c. Binding of GST-Nup153c to unphosphorylated mutant ERK2 proteins was decreased relative to binding to wild type ERK2 roughly in proportion to the decrease in their import. pY231A/L232A binds to GST-Nup153c less well than wild type pERK2. These results are consistent with the idea that the decreased import of certain ERK2 mutants is a result of their decreased interaction with the nuclear pore complex.

As an additional test of this idea, increasing concentrations of GST-Nup153c were added to the import assay. As GST-Nup153c concentration was increased, the import of GFP-ERK2 decreased. In contrast, addition of GST alone had no detectable effect on import (Fig. 5C).

Import of ERK2 Mutated in the D Domain Docking Site—Proteins with D motifs may influence the localization of ERK2 by anchoring it in one compartment or escorting it through the nuclear pore (21). Because ERK1/2 have no CRM1 consensus nuclear export sequence, CRM1-dependent export may occur by a piggyback mechanism, for example. We previously tested import of the ERK2 mutant D316A/D319A, which is defective in interactions with D motifs (19). This mutant was imported nearly as well as wild type ERK2 in the unphosphorylated (not shown) and the phosphorylated state (Fig. 4, A and B). Asp–319 was originally identified in Drosophila ERK2 in a screen for mutations that increased activity in a tyrosine kinase pathway (33). The adjacent residue Glu–320 (Glu–322 in human ERK2) was recently found mutated in a human cancer cell line, and its mutation was reported to result in constitutive activation of ERK2 (34). Thus, we prepared and tested this mutant in the import assay. ERK2 E320K and pERK2 E320K were imported indistinguishably from wild type ERK2 (Fig. 6A). In the unphosphorylated state ERK2 E320K had a significantly higher specific exchange study were mutated and tested in import assays. Both GFP-ERK2 I196A/M197A and N199A/S200A, predicted to interfere with Nup binding to ERK2, were impaired for import (Fig. 3A). Quantification of fluorescence intensity indicated close to 50% inhibition of nuclear entry for each mutant (Fig. 3C). Combinations of mutations did not further reduce import (data not shown). Residues Tyr–231 and Leu–232 on pERK2 were protected when bound to the Elk1 peptide. However, the behavior of the Y231A/L232A mutant in unphosphorylated proteins had a similar effect on the pERK2 import of pERK2 Y231A/L232A was decreased (Fig. 4, A and B). This would be expected if pERK2 binds Nups in a manner similar to substrate binding (32). The opposite occurred for ERK2 I196A/M197A (Fig. 4, A and B) and was consistent with the idea that these residues are involved in Nup binding independent of phosphorylation state. Other pERK2 mutants, K52R, the dimerization mutant L4A/H176E, and D316A/D319A, were imported like wild type pERK2 (Fig. 4, A and B). Together these results support the idea the Nups bind in the same vicinity of ERK2 as FXF motif-containing substrates, although the exact binding orientation varies with ERK1/2 phosphorylation state.

We showed previously that His6-ERK2 and the GFP-ERK2 fusion proteins behaved similarly in import assays despite the size difference (23). As a control for experiments here, we compared import of His6-ERK2 mutants using immunofluorescence. The behavior of pERK2 mutants in import assays was similar whether His6-tagged or GFP fusion proteins were examined (data not shown). To test for dephosphorylation of ERK2 and mutants by the residual phosphatase activities in the import assays, we used pERK1/2 antibodies to immunoblot the pERK2 proteins following incubation with permeabilized cells. Incubation of the phosphorylated proteins with digitonin-permeabilized cells for the time required for import assays revealed little difference in wild type or mutant pERK2 immunoreactivity (9) (data not shown), indicating that the proteins are not dephosphorylated during import assays.

FIGURE 5. In vitro binding of ERK2 and mutants to nucleoporin. A, binding of His6-ERK2 proteins to GST-Nup153c or GST. Bound and input proteins were detected with anti-ERK antibody Tyr-691. One of three similar experiments is shown. B, binding of phosphorylated His6-ERK2 and pERK2 Y231A/L232A to GST-Nup153c. Bound and input proteins were detected with anti-pERK antibodies. One of three similar experiments is shown. C, import of ERK2 in the presence of increasing concentrations of GST or GST-Nup153c. Fluorescent images of nuclei at selected time points are shown.
activity (15–20-fold) toward a model substrate than wild type ERK2 (Fig. 6B). As in the earlier report (34), we found that ERK2 E320K has a greater mobility than ERK2 in denaturing gels. Because pERK2 E320K had a specific activity similar to wild type pERK2 under our conditions, we did not characterize its activity further.

Import of ERK2 Mutated in the Vicinity of the Substrate Binding Site—We evaluated the import of ERK2 with mutations near the protein substrate binding pocket of the active site. ERK2 P224G, P227G, and W190A were tested. Mutation of either Pro-227 or Trp-190 was shown to have a significant impact on catalytic activity, causing a ~5-fold increase in $K_m$ and greater than a 30-fold increase in $k_{cat}/K_m$ for the substrate Elk1 (35). These mutants were each imported similarly to wild type ERK2 in either the unphosphorylated (Fig. 7, A and B) or the phosphorylated (Fig. 7, C and D) state.

Import of pERK2 Mutants in the Presence of Energy and Cytosol—Phosphorylated ERK2 can also enter the nucleus by an energy-dependent mechanism not available to the unphosphorylated protein (9). Although the relevant carriers have not yet been identified, this process is assumed to be dependent on cytosolic import carriers and not direct interactions with Nups. Thus, we also examined import of the pERK2 mutants in the presence of cytosol and an energy-regenerating system. All of the mutants were imported nearly as well as wild type ERK2 (Fig. 8, A and B). Minimal defects were observed for pERK2 L231A/L232A, Y261A, and I196A/I197A.

Evaluation of RanBP7 in MAPK Import—Nuclear transport factors influence the localization of MAPKs in other organisms. In Drosophila, importin7 (dim-7), interacts with fly ERK2 (36). Mutation of the gene encoding dim-7 caused decreased nuclear localization of Drosophila ERK2. To determine whether mammalian MAPKs may be imported via RanBP7, the human homolog of Dim7, we used RNA interference to knock down RanBP7 expression (Fig. 9A). The endogenous MAPKs ERK1/2, JNK, and p38 were activated normally by UV irradiation and phorbol 12-myristate 13-acetate in cells in which RanBP7 was
knocked down (Fig. 9B). No obvious changes in nuclear localization of any of these MAPKs were observed (Fig. 9, C–E). Thus, the energy-dependent import of ERK2 and the other MAPKs tested apparently takes place through the actions of some other import carrier.

**Localization of ERK2 Mutants in Cells**—ERK2 is distributed throughout the cell, in both cytoplasmic and nuclear compartments. In resting cells, as much as half of the inactive ERK2 may be in the nucleus (6), consistent with the energy-independent movement of ERK2 through the nuclear pore. Heterologous expression of ERK2 generally results in greater accumulation of ERK2 in the nucleus than observed with endogenous protein (15). We examined the distribution of ERK2 mutants that showed decreased import in vitro in HeLa or human embryonic kidney 293 cells. The majority of the mutants were found throughout the cell and in the nucleus (Fig. 10 and not shown), consistent with the localization of the endogenous protein and heterologously expressed wild type ERK2 in the absence of stimuli, as reported previously for some of the mutants (8, 37). The single exception was ERK2-(Δ241–272) (MAPK insert deletion); a reduced nuclear concentration of this ERK2 mutant was observed in most of the cells, in a few cases appearing as nuclear exclusion (Fig. 10).

**DISCUSSION**

The nuclear localization of ERK1/2 is highly regulated. We are using reconstitution of import and export in permeabilized cells to explore the mechanisms involved and to isolate the individual steps to elucidate their contributions to the behavior observed in intact cells. We have reconstituted import and export in several cell types. Although ligand-induced localizations of ERK1/2 are not identical in BJ and cell lines frequently used for localization studies (e.g. HeLa, Ref52), few distinctions among cell types have been observed in the import/export reconstitution experiments performed thus far. We selected BJ cells for the majority of these studies because they are immortalized in a defined manner, they are diploid, and, in contrast to several standard cell lines, different ligands induce distinct pERK1/2 localization patterns in BJ cells (38). Thus, we anticipate that a greater number of regulated steps can be discerned in this cell system.

We and others have previously found that ERK2 in the unphosphorylated state enters the nucleus by an energy-independent mechanism facilitated by direct interaction with nucleoporins (7, 23). This appears to be the primary entry mechanism for the unphosphorylated protein and a significant import mechanism for pERK2 as well. Consistent with this idea, free Nup153c inhibits the nuclear uptake of both unphosphorylated ERK2 and pERK2. Furthermore, nucleoporin binding is decreased for several ERK2 mutants that show a nuclear import defect in reconstitution assays.

From an examination of the crystal structures of ERK2, it seemed possible that unphosphorylated and phosphorylated forms of ERK2 might bind somewhat differently to hydrophobic sequences such as the FXF-rich motifs of Nups. Results of import and binding assays suggest that this may be the case (Table 1). Mutations in residues in the insert itself appeared to affect import of both unphosphorylated and pERK2, while other residues predominantly affect import of the unphosphorylated or the phosphorylated kinase. In this regard, we recently found that these ERK2 mutants bind less well to FXF motifs from the transcription factor substrate ERF (39). ERF contains four FXF motifs. One of these motifs binds better to unphosphorylated ERK2, whereas a second motif displays the selectivity for pERK2 that has often been found for FXF motifs. Together these results suggest that there is more than one mode through which ERK2 may interact with FXF motifs: the Elk peptide mode revealed by hydrogen-deuterium exchange using pERK2 (32), which is likely to be the common mode of interaction with protein substrates, and a second mode accessible primarily in the low activity state of ERK2.

Import reconstitution assays permit us to study individual processes in import and export that have distinct cofactor and interaction requirements. In addition, import and export can be measured over short times, which enables us to discern altered rates of import under defined conditions. In cells all import and export processes are available. Mutant proteins that are expressed have ample time to move throughout the cell using all of the existing processes before their localization is sampled. Thus, despite the fact that we can identify mutations that are defective in import using reconstitution assays, these mutations rarely impact the localization of ERK2 in cells. The permeabilized cell assays allow the discrimination of relatively subtle differences in protein properties.
Overexpression of ERK2 usually results in its relative nuclear accumulation in cells. This phenomenon has been attributed to overwhelming the capacity of cytoplasmic anchoring proteins and may also suggest a wealth of low affinity nuclear binding sites for ERK1/2. The majority of ERK2 mutants we expressed were distributed in both cytoplasmic and nuclear compartments and showed nuclear accumulation that was increased relative to endogenous protein (6, 38).

A puzzling observation is that ERK2-(Δ241–272) showed an increased cytoplasmic localization. This mutant is impaired in reconstitution assays. From earlier experiments this mutant interacts less well with MEK1/2 and is not phosphorylated by MEKs (8). Considerable evidence suggests that MEKs are key cytoplasmic anchoring proteins (40). Thus, we might have predicted that this protein would accumulate in the nucleus due to weakened cytoplasmic binding. This ERK2 deletion mutant is similar to a newly identified ERK1 splice form, ERK1c. The ERK1c splice form lacks an additional 12 residues, 11 of which are C-terminal to the residues deleted in the ERK2 mutant (41). Interestingly, ERK1c is localized to the Golgi. Further exploration of the localization mechanism of this protein may lead to some different ideas about why ERK2 accumulates in the nucleus as a default state.

Analysis of import of the deletion mutant and ERK2 Y261N in the presence and absence of energy revealed an unanticipated difference in import rate relative to wild type ERK2. Previously we found no difference in import of wild type, unphosphorylated ERK2 in the presence or absence of energy. Although the difference in behavior of ERK2 Y261N is small, it seems possible that it may reflect a subtle difference in import mechanism that was observed because of the slowed kinetics. This suggests the possibility of additional entry mechanisms for unphosphorylated ERK2 in addition to direct nucleoporin interactions.

A major component of nuclear entry of pERK2 is energy-dependent. Studies of ERK2 homologs in other organisms suggested that RanBP7 might be the relevant carrier in mammalian cells. However, RNA interference of RanBP7 had no effect on the localization of ERK2 or other MAPKs under stimulated conditions. It is possible that the assay conditions selected were inappropriate to measure the function of this protein in MAPK import. Alternatively, one or more other carriers may be the active import carrier in mammalian cells whose existence was inferred from our earlier transport studies (9).

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A puzzling observation is that ERK2-(Δ241–272) showed an increased cytoplasmic localization. This mutant is imported in permeabilized cells, although it is the most impaired in reconstitution assays. From earlier experiments this mutant interacts less well with MEK1/2 and is not phosphorylated by MEKs (8). Considerable evidence suggests that MEKs are key cytoplasmic anchoring proteins (40). Thus, we might have predicted that this protein would accumulate in the nucleus due to weakened cytoplasmic binding. This ERK2 deletion mutant is similar to a newly identified ERK1 splice form, ERK1c. The ERK1c splice form lacks an additional 12 residues, 11 of which are C-terminal to the residues deleted in the ERK2 mutant (41). Interestingly, ERK1c is localized to the Golgi. Further exploration of the localization mechanism of this protein may lead to some different ideas about why ERK2 accumulates in the nucleus as a default state.

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