Identification of a C-terminal Region That Is Required for the Nuclear Translocation of ERK2 by Passive Diffusion*

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Extracellular signal-regulated kinase 2 (ERK2) is located in the cytoplasm of resting cells and translocates into the nucleus upon extracellular stimuli by active transport of a dimer. Passive transport of an ERK2 monomer through the nuclear pore is also reported to coexist. We attempted to characterize the cytoplasmic retention and nuclear translocation of fusion proteins between deletion and site-directed mutants of ERK2 and green fluorescent protein (GFP). The overexpressed ERK2-GFP fusion protein is usually localized to both the cytoplasm and the nucleus unless a cytoplasmic anchoring protein is coexpressed. Deletion of 45 residues, but not 43 residues, from the C terminus of ERK2 prevented the nuclear distribution of the ERK2-GFP fusion protein. Substitution of a part of residues 299–313 to alanine residues also prevented the nuclear distribution of the ERK2-GFP fusion protein without abrogation of its nuclear active transport. These observations may indicate that the passive diffusion of ERK2 into the nucleus is not simple diffusion but includes a specific interaction process between residues 299–313 and the nuclear pore complex and that this interaction is not required for the active transport. We also showed that substitution of Tyr314 to alanine residue abrogated the cytoplasmic retention of the ERK2-GFP fusion protein by PTP-SL but not by MEK1.

Mitogen-activated protein kinase (MAPK)1 pathways include a family of protein serine/threonine kinases that are activated by a wide variety of extracellular stimuli, such as growth factors, hormones, cytokines, and cellular stresses (1, 2). Extracellular signal-regulated kinases 1 and 2 (ERK1/2), also known as p44/p42 MAPK, are well-studied members of this family, and it is known that they preferentially regulate cell growth and differentiation. Upon stimulation, ERKs are activated via a cascade of phosphorylation and activation of the protein kinases Raf-1 and MAPK/ERK kinase (MEK). Activated MEK, which is specific to ERKs, activates ERKs by catalyzing the phosphorylation of ERKs on both a threonine and a tyrosine residue. The activated ERKs can phosphorylate a variety of substrates, including transcription factors that control cellular growth, such as Elk-1.

In resting cells, ERKs are primarily localized to the cytoplasm. Upon extracellular stimulation, a fraction of cytoplasmic ERKs translocate into the nucleus (3). The dissociation of ERK2 from its cytoplasmic anchor is induced by the phosphorylation of ERK2 at Tyr (and Thr) (4–6). Phosphorylated ERK2 forms a homodimer and translocates into the nucleus (5–7). Because ERK2 does not have a clear nuclear localization signal, it is hypothesized that this dimerization provokes the nuclear localization signal. However, the nuclear translocation mechanism of ERK2 still remains to be clarified. Nuclear-transported ERK2 is dephosphorylated and exported to the cytosol by a Crm1-dependent mechanism that involves MEK1 (8). Adachi et al. (6) have demonstrated that there is another nuclear import system of ERK2 different from the above-mentioned active transport and that this transport is driven by passive diffusion of a monomer.

Recent studies have shown that several kinds of protein are sufficiently robust to anchor ERK2 protein in the cytoplasm when they are overexpressed. These proteins include MEK1 (9), dual specificity MKP3 (a MAPK phosphatase specific for ERK) (10), and the cytoplasmic part of PTP-SL (a protein-tyrosine phosphatase) (11). These interaction partners of ERK2 have been demonstrated to have docking domains recognized by other MAPKs in a similar way. These docking domains are conserved and characterized by the presence of a cluster of basic residues (12–18). For instance, the sequences LXR-XRXRXRXL of PTP-SL and KKKKXXXL in the N-terminal region of MEK1 have been shown to be required for their bindings to ERK2. As the counterpart of these positively charged amino acid residues, a motif containing two acidic residues, Asp316 and Asp319, for the binding was identified in the region of MEK1. Studies using chimera proteins from ERK2 and p38 MAPK have indicated that the major C-terminal structural lobe is the binding site of ERK2 for MKP3 (20) and that a MEK1-binding site of ERK2 is localized to its N terminus (21–23).

In the present study, we examined the role of the C-terminal region of ERK2, which contains the CD domain, for its subcellular localization and nuclear translocation. We found that Tyr314 near the CD domain is important for the binding between ERK2 and PTP-SL. Overexpressed ERK2 is usually dis-
tributed in both the cytoplasm and the nucleus unless a cytoplasmic anchoring protein is coexpressed. Interestingly, we found that some C-terminal deletion mutants of the ERK2 fusion protein were localized to the cytoplasm even in the absence of exogenously expressed anchoring proteins and identified the relevant sequence.

**EXPERIMENTAL PROCEDURES**

**Subcloning and Mutagenesis—**Wild-type mouse ERK2 and its mutants were ligated into the pEGFP-N3 vector (CLONTECH) and into the site downstream of the GFP gene of the pEGFP-C1 vector, which was constructed in our laboratory. The deletion and site-directed mutants of ERK2 were constructed by PCR-based mutagenesis, and the mutations were confirmed by DNA sequencing. cDNAs for expressing wild-type mouse MEK1 and FLAG-tagged human PTP-SL (147-549/C480S) (11) were cloned into the pReCMV vector (Invitrogen). The expression of these proteins was confirmed by immunoblotting after separation by SDS-PAGE. In the case of (1-315)-GFP, the ERK2 mutant was cloned into the site upstream to three tandemly connected GFPs. The mutants used in this study are listed in Tables I and II.

**Antibodies and Reagents—**A mixture of two mouse monoclonal anti-GFP antibodies (clones 7.0 and 13.1) was obtained from Roche Diagnostics. Anti-dual phosphorylated ERK antibody and anti-MEK1 antibody were obtained from New England Biolabs, Inc. Anti-Flag M5 monoclonal antibody was purchased from Sigma. Leptomycin B and tetradercanoyl phorbor acetate (TPA) were obtained from Sigma. Phe- nylosine oxide was from Wako Chemicals.

**Cell Cultures, Transfection, and Localization Studies—**Chinese hamster ovary (CHO) cells (obtained from Japanese Cancer Research Resources Bank) were grown in Ham’s F-12 medium containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) at 37 °C in an atmosphere of 5% CO2. CHO cells were seeded 105 cells/well in a 6-well plate, cultured for 24 h, and transfected with 0.5 μl of total DNA using 6 μl of FuGene 6 (Roche Diagnostics) according to the manufacturer’s instructions. In cotransfection, two kinds of plasmids were introduced at a ratio of 1:1 (w/w) unless otherwise noted. Localization of GFP-fused protein was visualized using conventional fluorescence microscopy (Nikon ECLIPSE TE300). The results were usually observed in more than 80% of the cells expressed.

**Preparation of Cell Extracts, Immunoprecipitation, and Immunoblotting—**For detecting the phosphorylation state of ERK2, cells transfected with expression plasmids were stimulated with peroxovanadate (VOOH, 100 μM Na3VO4, 200 μM H2O2) for 15 min and lysed, and the proteins were solubilized with hot SDS solution containing 1% SDS, 1 mM Na3VO4 (Calbiochem), and 10 mM Tris, pH 7.4.

For ERK2-PTP-SL (147-549/C480S) immunoprecipitation experiments, the cell cultures were washed twice with ice-cold phosphate-buffered saline, and the cells were lysed in lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 2 mM Na3VO4) and incubated on ice for 30 min. The lysates were centrifuged for 10 min at 15,000 rpm, and the ERK2-GFP fusion proteins were immunoprecipitated from the supernatant with anti-FLAG M2 Affinity Gel beads (Sigma). The beads were washed three times in 20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, and 0.1% Triton X-100, followed by SDS-PAGE.

For immunoblotting, the proteins were separated by a 10% SDS-PAGE, transferred onto a polyvinylidene fluoride membrane, and probed with appropriate antibodies. The binding of antibodies was detected using ECL (Amersham Biosciences).

**RESULTS**

**Deletion of a C-terminal Region of ERK2 Causes the Cytoplasmic Localization of ERK2-GFP Fusion Protein—**To characterize the subcellular localization of ERK2, we constructed a plasmid that expresses a C-terminally truncated ERK2 protein in which the C-terminal 153 amino acids (GFP) were removed, termed ERK2-GFP. In this way, the formation of GFP chromophore is directly related to the correct folding of the upstream protein (24). The plasmid was transfected into CHO cells and was examined to determine whether the subcellular localization of ERK2-GFP is different from that of wild-type ERK2 itself under various conditions. As described before (19), the expression of the ERK2-GFP showed an essentially similar subcellular localization and intracellular movement upon mitogenic stimulation as ERK2 itself (Fig. 1A).

The intact ERK2-GFP fusion protein was distributed in both the cytoplasm and the nucleus in the absence of coexpressed MEK1. When MEK1 was coexpressed, ERK2-GFP was excluded from the nucleus. TPA and leptomycin B treatments induced the ERK2-GFP translocation into the nucleus. The same was true for ERK2- (GFP)5. Taken together, all of these characteristics were not apparently distinguishable from those of wild-type ERK2.

In quiescent cells endogenous ERK2 predominantly localizes to the cytoplasm by binding to cytoplasmic anchors including MEK. However, overexpressed ERK2 usually localizes to both the cytoplasm and the nucleus, because an excess amount of ERK2 over MEK diffuses into the nucleus (6). With this in mind, to investigate whether the C-terminal amino acid se-
quency of ERK2 is important for its cytoplasmic localization, we constructed C-terminal deletion mutants of the ERK2-GFP fusion protein. These mutants were expressed in CHO cells without coexpressed MEK1 (Fig. 1B, left column). Up to (1–318)-GFP, which contains residues 1–318 of ERK2, the mutants showed subcellular localization similar to that of (1–358)-GFP (full-length ERK2-GFP). Interestingly, further deletion of 10 amino acid residues from (1–318)-GFP caused the cytoplasmic localization of the mutant, (1–308)-GFP. To eliminate the possibility that the position of GFP may influence the subcellular localization of these mutants, we also constructed plasmids in which GFP was fused to the N terminus of the ERK2 deletion mutants, GFP-(1–338), GFP-(1–328), GFP-(1–318), and GFP-(1–308). When expressed in CHO cells, these GFP-ERK2 deletion mutants displayed similar localization in the cells as a series of ERK2-GFP deletion mutants (Fig. 1B, right column). Thus, the cytoplasmic localization of (1–308)-GFP was independent of the position of GFP.

Furthermore, to characterize the mechanism of this cytoplasmic localization, we constructed GFP fusion proteins containing residues 1–313 and residues 1–315 of ERK2. When expressed in CHO cells, whereas (1–313)-GFP was retained in the cytoplasm of ~80% of the expressed cells, (1–315)-GFP, which contained two additional tyrosine residues, was distributed throughout the cell (Fig. 1C). Thus, the deletion of two extra tyrosine residues, Tyr314 and Tyr315, caused an obvious difference in the subcellular localization of the mutants. Fig. 1D shows the phosphorylation state of the ERK2-GFP deletion mutants. Immunoblotting analysis indicated no incorporation of phosphates into the regulatory sites of the (1–315)-GFP, (1–313)-GFP, and (1–308)-GFP fusion proteins upon VOOH stimulation. Because the phosphorylation at these regulatory residues is requisite for the dissociation of ERK2 from MEK1 and the active nuclear translocation of ERK2, it was suggested that the nuclear distribution of (1–315)-GFP was not because of phosphorylation-dependent nuclear translocation. In addition, we determined whether the nuclear distribution of (1–315)-GFP was through size-dependent passive diffusion by examining the subcellular localization of (1–315)-(GFP), a plasmid expressing ERK2 (1–315) connected to three GFPs in series. The molecular mass of (1–315)-(GFP) was ~115 kDa, which is considered above the diffusion limit of the nuclear pore. In more than half of the cells expressed, (1–315)-(GFP) was localized to the cytoplasm (Fig. 1C). This result suggests that the nuclear distribution of (1–315)-GFP may be at least partly due to size-dependent simple diffusion through the nuclear pore. We next tested whether (1–313)-GFP aggregated as an insoluble form in the cytoplasm. The cells transfected with (1–313)-GFP were treated with 40 μM of phenylarsine oxide, which is known to induce nuclear translocation of ERK1 without its activation (25). As shown in Fig. 1C, phenylarsine oxide treatment induced (1–313)-GFP to distribute in both the cytoplasm and the nucleus, indicating that (1–313)-GFP is diffusible. Taken together, these results indicate that (1–315)-GFP translocates into the nucleus by passive diffusion, but (1–313)-GFP is unable to enter the nucleus by passive diffusion despite its smaller size, suggesting that translocation by diffusion through the nuclear pore requires interaction between the ERK2-GFP fusion protein and the nuclear pore complex.

Point Mutations of ERK2 Cause the Cytoplasmic Localization of ERK2-GFP Fusion Protein—To investigate whether Tyr<sup>314</sup> and Tyr<sup>315</sup> are important for the passive diffusion of the ERK2 fusion protein, we then constructed (1–318)5A-GFP, (1–328)5A-GFP, (1–338)5A-GFP, (1–348)5A-GFP, and (1–358)5A-GFP, in all of which residues 314–318 were mutated to alanine residues (Table I). When these mutants were expressed in CHO cells, there were minimal changes in the subcellular localization (data not shown), suggesting that Tyr<sup>314</sup> and Tyr<sup>315</sup> are not necessary for the passive diffusion process and that the cytoplasmic localization of (1–313)-GFP was not due to the lack of these two tyrosine residues.

Because the mutation of the sequence including Tyr<sup>314</sup> and Tyr<sup>315</sup> caused no cytoplasmic localization of the fusion proteins, it was considered that deletion of residues 314–358 might influence the conformation of the other region of ERK2. Therefore, based on the ERK2 crystallographic data (26), we mutated the residues closely surrounding Tyr<sup>314</sup> and Tyr<sup>315</sup> and examined the cytoplasmic localization of these ERK2-GFP mutants (Fig. 2 and Table I). When expressed in CHO cells in the absence of exogenous MEK1, mutants 119A3-GFP, 122A3-GFP, 125A6-GFP, and 296A3-GFP were distributed throughout the cell (Fig. 3A, left column). 119A3-GFP, 122A3-GFP, and 296A3-GFP were retained in the cytoplasm with exogenous MEK1, and phosphorylated at both Thr<sup>383</sup> and Tyr<sup>385</sup> upon VOOH stimulation, displaying similar characteristics to those of the wild-type ERK2-GFP fusion protein (Fig. 3B). 125A6-GFP lost its capability to be phosphorylated by MEK1. 299A3-GFP, 302A6-GFP, 308A3-GFP, and 311A3-GFP displayed the cytoplasmic localization even in the absence of exogenous MEK1, as in the case of (1–313)-GFP (Fig. 3A, right column). Of these mutants, 302A6 and 311A3 were induced to translocate into the nucleus by TPA stimulation for 1 h (Fig. 3A, right column). In accordance with these results, VOOH stimulation phosphorylated 302A6-GFP and 311A3-GFP at both of the regulatory sites (Fig. 3B). On the other hand, 299A3-GFP and 308A3-GFP were not phosphorylated and also inactive with respect to nuclear translocation by TPA stimulation (Fig. 3A, right column). The effect of leptomycin B, which has been shown to specifically inhibit nuclear export signal-mediated active nuclear export of proteins including MEK1 (9, 27), was examined on subcellular localization of 299A3-GFP, 302A6-GFP, 308A3-GFP, and 311A3-GFP. After treatment with 8 ng/ml of leptomycin B for 1.5 h, there was minimal change in the distribution of both 299A3-GFP and 308A3-GFP, whereas 302A6-GFP was distributed throughout the cell, indicating a leakage of the mutant probably because of its active transport mechanism. The nuclear distribution of 311A3-GFP after treatment with leptomycin B was observed in a small portion of the cells. These results indicate that the cytoplasmic localization of

### Table I

| Name Mutations | ERK2 mutants used in this study |
|---------------|--------------------------------|
| (1–338) deletion of residues 339–358 |
| (1–328) deletion of residues 329–358 |
| (1–318) deletion of residues 319–358 |
| (1–315) deletion of residues 316–358 |
| (1–313) deletion of residues 314–358 |
| (1–308) deletion of residues 309–358 |
| 119A3 L119A, S120A, and N121A |
| 122A3 D122A, H123A, and N124A |
| 125A6 C125A, Y126A, F127A, L128A, Y129A, and Q130A |
| 296A3 P296A, H297A, and K298A |
| 299A3 R299A, I300A, and E301A |
| 302A6 V302A, E303A, Q304A, and L306A |
| 308A3 H308A, P309A, and Y310A |
| 311A3 L311A, E312A, and Q313A |
| 5A Y314A, Y315A, D316A, P317A, and S318A |
| (1–348)5A deletion of residues 349–358, Y314A, Y315A, D316A, P317A, and S318A |
| (1–338)5A deletion of residues 339–358, Y314A, Y315A, D316A, P317A, and S318A |
| (1–328)5A deletion of residues 329–358, Y314A, Y315A, D316A, P317A, and S318A |
| (1–318)5A deletion of residues 319–358, Y314A, Y315A, D316A, P317A, and S318A |
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Fig. 2. Sites of mutations and deletions made in ERK2. Three-dimensional structure of ERK2. Tyr^{314} and Tyr^{315} are shown in red, residues 299–313 are shown in yellow, residues 119–130 and 296–299 are shown in cyan, and residues 316–358 are shown in green. The figure was generated using the RasMol program, based on the crystallographic data (26).

Fig. 3. Cytoplasmic localization of ERK2-GFP mutants in CHO cells. A, CHO cells were transfected with each of 119A3-GFP, 122A3-GFP, 125A6-GFP, and 296A3-GFP together with or without plasmid expressing MEK1. CHO cells were also transfected with each of 299A3-GFP, 302A6-GFP, 308A3-GFP, and 311A3-GFP, and then the cells were treated with 250 nM TPA for 1 h and 8 ng/ml leptomycin B (LB) for 1.5 h. B, CHO cells were transfected with each of the following constructs: ERK2-GFP (WT), 119A3-GFP, 122A3-GFP, 125A6-GFP, 296A3-GFP, 299A3-GFP, 302A6-GFP, 308A3-GFP, and 311A3-GFP. The cells were serum-starved for 6 h and then were either left untreated (−) or stimulated with VOOH (+). The cell lysates were prepared, separated on an SDS-PAGE, and blotted onto polyvinylidene fluoride membrane. The membrane was analyzed with anti-dual phosphorylated ERK antibody (Anti-P-ERK) or anti-GFP antibody (Anti-GFP). Each of these experiments was reproduced at least three times.

Table II

| Name       | Mutations                        |
|------------|----------------------------------|
| YY33A      | Y314A, Y315A, and S318A          |
| Y314A      | Y314A                            |
| Y315A      | Y315A                            |
| D316A      | D316A                            |
| S318A      | S318A                            |
| D319A      | D319A                            |

dependent active transport. Thus, suppression of the passive diffusion into the nucleus of these overexpressed mutants is not because of increased interaction between MEK and ERK and is independent of the active transport mechanism. It was also indicated that residues 299–313 are required for the passive diffusion of ERK2-GFP into the nucleus.

Ty^{314} of ERK2 Is Required for Its Binding to PTP-SL—To determine the role of Ty^{314} and Ty^{315} for the binding capability of ERK2 to anchoring proteins, we also constructed plasmids expressing full-length ERK2-GFP fusion proteins with mutations at these tyrosine residues and their adjacent residues (Table II) and tested whether the exogenous intracellular region (residues 147–549) of PTP-SL and MEK1 would be able to retain these mutants in the cytoplasm. As reported previously (11), coexpressed PTP-SL retained the full-length ERK2-GFP fusion protein as MEK1 does (Fig. 4A). When YY33A-GFP was expressed in CHO cells without exogenous anchoring proteins, this mutant distributed in both the cytoplasm and the nucleus. Exogenous PTP-SL did not retain the mutant in the cytoplasm, whereas exogenous MEK1 excluded the mutant from the nucleus. The ERK2-GFP mutant in which residues 313–318 were all mutated to alanine residues showed the same distribution as YY33A-GFP (data not shown). Furthermore, to clarify the role of the respective amino acid residues, we also examined whether Y314A-GFP, Y315A-GFP, D316A-GFP, S318A-GFP, and D319A-GFP were retained in the cytoplasm by PTP-SL and MEK1. Asp^{316} and Asp^{319} have been reported to be important for the interaction between ERK and MEK by binding to positively charged residues in the docking domain (13, 19). The crystallographic data of ERK2 has shown that Ser^{318} is clustered with Asp^{316} and Asp^{319} on the surface of the protein (26). Y315A-GFP, D316A-GFP, and S318A-GFP were retained in the cytoplasm by exogenous PTP-SL, although neither Y314A-GFP nor D319A-GFP was retained. These findings indicate that residues Tyr^{314} and Tyr^{315} for the binding capability of ERK2 with PTP-SL and that acidic residue Asp^{316} is not required for the cytoplasmic retention of ERK2 by PTP-SL. Fig. 4A also shows that MEK1 coexpression induces cytoplasmic localization of all of these mutants, and these results may indicate that a region in addition to the CD domain contributes to the cytoplasmic retention of ERK2 by MEK1.

Immunoprecipitation analysis supported the above-mentioned results (Fig. 4B). Here, we tested the ability of the ERK2-GFP mutants to coimmunoprecipitate with PTP-SL. FLAG-tagged PTP-SL was coexpressed with each of the ERK-GFP mutants in CHO cells, and the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody conjugated with agarose beads. Immunoblotting with anti-GFP antibody demonstrated that the full-length ERK2-GFP fusion protein, Y315A-GFP, and S318A-GFP showed strong coimmunoprecipitation with PTP-SL and that D316A-GFP showed a slightly decreased ability to associate with PTP-SL. Almost no association of PTP-SL with YY33A-GFP, Y314A-GFP, or D319A-GFP was detected by anti-GFP antibody under the present conditions. These results indicate that the mutation of Ty^{314} and Asp^{319} abolished the ability of ERK2 to associate with PTP-SL, resulting in the loss of its cytoplasmic retention.
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In resting cells, ERK2 is retained in the cytoplasm by anchoring proteins, and phosphorylation of ERK2 upon stimulation induces its homodimerization and promotes its accumulation in the nucleus by active transport mechanism (5, 7). Recently, it has been demonstrated that Xenopus MAPK diffuses into the nucleus as a monomer (6). The present study showed that the deletion of 45 residues from the C terminus of ERK2 caused the cytoplasmic localization of the ERK2-GFP fusion protein. This mutant was considered to be unable to diffuse into the nucleus despite its small size, which makes it possible to penetrate the nuclear pore, indicating that passive diffusion into the nucleus may require a sequence recognizable by the nuclear pore complex. In this study, we also identified residues 299-RIEVEQALAHPYLEQ313 of ERK2 as the sequence necessary for its passive diffusion into the nucleus. This region forms α-helix i (26) and is located near the CD domain (Fig. 2). This may indicate that the binding of a protein containing a docking domain to ERK2 could restrict the passive diffusion of ERK2, even if the protein complex is small enough to pass through the nuclear pore. Recently, Matsuhashi et al. (28) suggested that the GFP-MAPK fusion protein passes through the nuclear pore by interacting with FG repeat of nucleoporin CAN/Nup214 by an energy-independent mechanism. It has been indicated that FG repeats serve as docking sites for transport receptors and likely interact with hydrophobic pockets running along the outside of the karyopherin (29, 30). Although the sequence we identified in this study appears not to form an obvious hydrophobic patch, the sequence may be involved in this process.

In the mutants of which the diffusion into the nucleus is restricted, there were two types of cytoplasmic localization. One is the type where the mutants cannot be phosphorylated or translocated into the nucleus upon mitogenic stimulation. These mutants are considered to be unable to properly associate with MEK1, and consequently the Crm1-dependent export system does not work as a nuclear exporter for these mutants. The other is the type where the mutants can interact with MEK1 and translocate into the nucleus upon stimulation, indicating that they can form a homodimer and interact with the nuclear pore complex directly or indirectly. Thus, passive diffusion and active transport of ERK2-GFP may use different binding sites of the nuclear pore complex to enter the nucleus. Although the physiological significance of the cytoplasmic localization of the mutants remains to be clarified, it is surmised that passive diffusion of ERK2 into the nucleus might be prevented under certain conditions, and this prevention might affect the MAPK signal transduction pathway.

The present study showed that substitution of Tyr314 to alanine residue in ERK2-GFP abrogated its cytoplasmic retention by PTP-SL and its association with PTP-SL. In contrast, no significant changes between the mutant and the wild type were detected in the cytoplasmic retention by MEK1. It has been reported that a leucine residue and two hydrophobic residues upstream and downstream from the basic residues in the docking domain of PTP-SL, respectively, are important for the association of PTP-SL with ERK1/2 (11). MEK1 lacks the corresponding upstream leucine residue. Recently, Xu et al. (31) reported that Tyr314 and Tyr315 on ERK2 play roles in docking with MEK1 and that the two tyrosine residues may interact with the above-mentioned hydrophobic residues. Our results suggest that Tyr315 may be unnecessary for the interaction of ERK2 with other proteins, and that the mutation at Tyr314 does not significantly influence the cytoplasmic retention of ERK2 by MEK1. Because the major difference in the docking domain of PTP-SL from that of MEK1 is the leucine residue upstream from the basic residues, Tyr314 may interact with this leucine residue, resulting in the contribution to stability of the kinase-phosphatase complex observed.

The CD domain is pivotal for recognizing the positively charged region found in the docking domains. However, the specificity of the interaction is unable to be explained by only these acidic residues. PTP-SL recognizes two different classes of MAPKs, ERK and p38 MAPK, but not c-Jun N-terminal kinase 1 (11). Because the two acidic residues are conserved through all of the MAPK families, the interaction partners of ERK2 appear to simultaneously recognize sites other than these acidic residues. The corresponding sequences in p38α MAPK and c-Jun N-terminal kinase 1 to the 314YYDPSD319 sequence of ERK2 are YHDPDD and WYDPSE, respectively. Thus, the difference between Tyr and Trp might contribute to the binding and functional specificity. To extend the understanding of the mechanism of substrate binding to MAPKs, detailed work on the specificity of determinants will be required.

The present study also showed that whereas the D319A mutation abrogated both the cytoplasmic retention of ERK2-GFP by PTP-SL and the association between them, substi-
tion of Asp$^{316}$ to an alanine residue had no influence on the cytoplasmic retention by PTP-SL despite a measurable decrease of coprecipitation of (D316A)-GFP with PTP-SL, indicating that the role of Asp$^{316}$ for the retention of ERK2 in the cytoplasm by PTP-SL may not be so essential under the conditions used in this study. In contrast, MEK1 retained all of the mutants examined in the cytoplasm. These cytoplasmic retentions might be rationalized by the postulation that MEK1 is able to associate with these mutants, although the interaction between them is weak, and has the ability to export the mutants from the nucleus. On the other hand, a MEK1 mutation study demonstrated that both the hydrophobic and basic residues are necessary for MEK1 to exclude overexpressed ERK2 from the nucleus in unstimulated cells (31). Recently, Robinson et al. (32) identified ERK2 point mutants that failed to interact with MEK1 in a two-hybrid screen. These mutants retained the ability to interact with MAPK-interacting kinase 1, MKP3, and a docking site from ribosomal S6 protein kinase, which are bound to ERK2 through their docking domains. Thus, the presence of additional binding sites to the CD domain is suggested in the case of interaction with MEK1.

Finally, we have shown that Tyr$^{314}$ of ERK2 is essential for its interaction with PTP-SL and that the region close to this residue is important for the passive diffusion of ERK2 into the nucleus. These results raise the possibility of regulatory significance of Tyr$^{314}$ for the passive diffusion into the nucleus.

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