Identification of Novel Point Mutations in ERK2 That Selectively Disrupt Binding to MEK1*

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Extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) are essential components of pathways through which signals received at membrane receptors are converted into specific changes in protein function and gene expression. As with other members of the mitogen-activated protein (MAP) kinase family, ERK1 and ERK2 are activated by phosphorylations catalyzed by dual-specificity protein kinases known as MAP/ERK kinases (MEKs). MEKs exhibit stringent specificity for individual MAP kinases. Indeed, MEK1 and MEK2 are the only known activators of ERK1 and ERK2. ERR2-MEK1/2 complexes can be detected in vitro and in vivo. The biochemical nature of such complexes and their role in MAP kinase signaling are under investigation. This report describes the use of a yeast two-hybrid screen to identify point mutations in ERK2 that impair its interaction with MEK1/2, yet do not alter its interactions with other proteins. ERR2 residues identified in this screen are on the surface of the C-terminal domain of the kinase, either within or immediately preceding α-helix G, or within the MAP kinase insert. Some mutations identified in this manner impaired the two-hybrid interaction of ERK2 with both MEK1 and MEK2, whereas others had a predominant effect on the interaction with either MEK1 or MEK2. Mutant ERK2 proteins displayed reduced activation in HEK293 cells following epidermal growth factor treatment, consistent with their impaired interaction with MEK1/2. However, ERK2 proteins containing MEK-specific mutations retained kinase activity, and were similar to wild type ERK2 in their activation following overexpression of constitutively active MEK1. Unlike wild type ERK2, proteins containing MEK-specific point mutations were constitutively localized in the nucleus, even in the presence of overexpressed MEK1. These data suggest an essential role for the MAP kinase insert and residues within or just preceding α-helix G in the interaction of ERK2 with MEK1/2.

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The abbreviations used are: ERK, extracellular signal-regulated kinases; MEK, MAP kinase kinase/ERK kinase; MAP, mitogen-activated protein; GAD, Gal4 transcriptional activation domain; RSK, ribosomal S6 kinase; CSM, complete supplemental medium; HEK, human embryonic kidney; EGF, epidermal growth factor; GST, glutathione S-transferase; MBP, myelin basic protein; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid.
are located on the opposite surface of ERK2 from the activation loop. This site has been termed the common docking (CD) domain (21). Hydrophobic amino acids, on the surface of ERK2 and immediately adjacent to Asp316 also play a role in MEK1-ERK2 docking (22). The CD domain plays an important role in the binding of D domain-containing proteins to MAP kinases. However, additional protein elements within ERK2 play essential roles in binding to MEK1. Here we used a two-hybrid screen of an ERK2 mutant library to identify residues specifically involved in the interaction with MEK1/2.

**EXPERIMENTAL PROCEDURES**

**Plasmid DNA Constructs:** Construction of pGAD-ERK2—The wild type rat ERK2 cDNA was PCR amplified using oligonucleotides FRO-5 (GGGCGGATCCCATATGGGCGCGCCGCGC) and FRO-9 (CGG-GGCTCGAGTGATCCTTGCTCGGCTGGAATCG) and the resulting DNA fragment was digested with BamHI and XhoI and ligated into pGAD-GH (30) which had been cut with the same enzymes. The resulting construct encodes full-length ERK2 fused to the C terminus of the Gal4 transcriptional activation domain (GAD). Two-hybrid vectors encoding LexA fusion proteins were generated by PCR amplification specific cDNAs with 5’ and 3’ primers that introduced BamHI (5’) and XhoI (3’) sites flanking the various coding sequences. The resulting DNA fragments were digested with BamHI and XhoI and ligated into pJVL11 (31) that had been digested with BamHI and SstI. The resulting constructs encode the various fusion proteins to the C terminus of the LexA DNA-binding domain. Mammalian expression vectors encoding triple FLAG epitope-tagged ERK2 and ERK2 mutants were constructed by digesting pGAD-ERK2 with BamHI and XhoI and ligating the resulting DNA fragment into pXFLAG-CMV7.1 (Sigma number E4026), which had been digested with BglII and SacI. All plasmid constructs were sequenced to confirm that spurious mutations were not introduced.

**Yeast Two-hybrid Experiments—**Pairwise interaction tests were carried out as follows. The yeast strain L40 (32) was co-transformed with a combination of LexA and GAD plasmids to be tested, five independent transformants were isolated and observing for growth. The expression of all LexA and GAD fusion sequences from human MEK1, human MEK2, human MNK1, rat MNK2, and chicken RSK. Co-transformants were selected by plating cells on complete supplemental medium (CSM) (BIO 101, Inc., number 4550-522) lacking Leu and Trp. Protein-protein interactions were tested by streaking co-transformed isolates on CSM lacking His, Leu, and Trp and observing for growth. 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.

**Semiquantitative Yeast Two-hybrid Interaction Assays—**For each combination of LexA and GAD plasmids to be tested, five independent yeast colonies were simultaneously used to inoculate 10 ml of liquid CSM-Leu-Trp medium. Such cultures were incubated overnight at 30 °C. Yeast cells equivalent to 1.5 ml of a 1.5 × 10^9 cell/ml culture were collected by centrifugation, washed once with 1 ml of LaciZ buffer (100 mM Na-phosphate, pH 7.5, 10 mM KCI, 1 mM MgSO_4_, and resuspended in 300 μl of LaciZ buffer. This mixture was frozen using liquid nitrogen, thawed, and split into three 100-μl aliquots. To each aliquot, 681 μl of LaciZ buffer and 19 μl of β-mercaptoethanol were added. Reactions were initiated by the addition of 160 μl of β-nitrophenyl β-D-galactopyranoside substrate (a 4 mg/ml solution in LaciZ buffer), incubated for 15 min at 37 °C and terminated with the addition of 400 μl of 1 N Na_2CO_3_. Insoluble material was removed by microcentrifugation and the amount of reaction product was quantified by measuring the absorbance of the supernatant at 420 nm. Under these assay conditions, 420-nm absorbance was linear with respect to the ATPase activity over a range of A_{420} values from <0.065 to 2.27 (data not shown).

**Construction of a Mutant ERK2 Two-hybrid Library—**The full-length ERK2 cDNA was mutagenized by low-fidelity PCR using Taq DNA polymerase, pGAD-ERK2 was amplified using primers FRO-30 (GAGATCCTAGAAGTACGTTGTTACCC) and FRO-31 (GGG-TACGCGGCCGCGCGGCGG) and cloned into pGAD-GH that had been digested with BamHI and XhoI and the ligation products were used to transform Esherichia coli. The resulting mutant library contained 2 × 10^6 potentially unique ERK2 cDNAs and its predicted mutation rate was such that 65% of the cDNAs should have contained a single mutation.

**Yeast Two-hybrid Screen for Mutant ERK2 Proteins Losing Interaction with MEK1—**Similar screens have been used to identify target-specific mutations in the p21 Ras GTPase (34). The pGAD-ERK2 mutant library was transformed into yeast strain AMR70 (MATa) (32) that had been transformed with either pLexA-MEK1-K97M or pLexA-MNK1. Diploids were selected by plating onto CSM-Leu-Trp-Leu plates and protein-protein interactions were then transferred into diploids onto CSM-His-Leu-Trp plates and observing for growth. MEK1-specific ERK2 mutants were identified as those which failed to grow on CSM-His-Leu-Trp medium when co-transformed with pLexA-MEK1-K97M, but grew normally when co-transformed with pLexA-MNK1. Approximately 10° yeast colonies were screened in this manner. Yeast colonies having a MEK1-specific mutant interaction profile were isolated from the original CSM-Leu library plates and their mutant pGAD-ERK2 plasmids were isolated and used to transform E. coli. Plasmid DNA was then prepared and mutated ERK2 cDNAs were sequenced.

**Cell Culture, Transfection, Cell Lysis, and Immunoblotting—**Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were cultured at 60–80% confluence in 60-mm dishes by adding 0.45 ml of a calcium phosphate precipitate, containing 1.4 μg of each plasmid DNA, to the 5 ml of culture medium. Transfection efficiency was between 30 and 50%. After 16 h of incubation, the precipitate-containing medium was removed and replaced with serum-free Dulbecco’s modified Eagle’s medium. 24 h later, cells were treated with epidermal growth factor (EGF) (50 ng/ml) for 5 min. The culture medium was then removed and the cells were lysed in 0.4 ml of lysis buffer (50 mM Tris, pH 7.6, 0.15 mM NaCl, 0.5% Triton X-100, 0.1 mM NaF, 1 μM pepstatin A, 1 μM leupeptin, 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10.5 μM aprotinin) and microcentrifuged for 5 min. 5–12 μl of the resulting cleared lysate was resolved by SDS-PAGE (10% gels) and transferred to nitrocellulose membranes. Antibodies specific for phospho-ERK1/2 (BOSTER SOURCE. Int. number 44-680), total ERK1/2 (Y691 serum) (35) or MEK1 (Santa Cruz number SC-219) were used for immunoblotting as described in Ref. 36.

**Immunoprecipitation and Protein Kinase Assays—**Four μg of anti-FLAG M2 antibody (Sigma number F3165) was added to 0.2 ml of cell lysate and incubated at 4 °C for 1 h. 30 μl of a 50% slurry of Protein A-Sepharose CL-4B (American Bioscience number 17-0963-03) in lysis buffer was added and this mixture was incubated at 4 °C for 1 h. Immune complexes were washed three times with 1 ml of 1 mM NaCl, 20 mM Tris (pH 7.4) followed by once in 10 mM Hepes (pH 8.0), 10 mM MgCl_2_. The drained beads were resuspended in a 30-μl kinase reaction mixture (10 mM Hepes, pH 8.0, 10 mM MgCl_2_, 50 μM ATP, 1 mM dithiothreitol, 1 mM benzamidine), which contained 10 μM of γ-32P]ATP and 5 μg of GST-G2ks containing residues 1–148 of human MEK2. Kinase reactions were incubated for 30 min at 30 °C, placed on ice, and terminated by adding 7.5 μl of 5 × SDS-PAGE sample buffer and boiling for 3 min. Twenty μl of this mixture was resolved by SDS-PAGE (10% gels). Gels were fixed, Coomassie-stained, dried, and used for autoradiography. Gel bands corresponding to the GST-Myc substrate were excised and their radioactivity determined by scintillation counting. MEK1 and MEK2 immunoprecipitations were performed as above except that antibodies A2227 (MEK1-specific) and A2228 (MEK2-specific) (37) were used. MEK1/2 kinase assays were carried out as those described for ERK2 except that 5 μg of GST-ERK2-K52R protein was used as substrate.

**Kinetic Analysis of Mutant ERK2 Proteins—**His6-tagged ERK2 and ERK2 mutants were expressed in E. coli using the NpT75-His-ERK2 construct (38) and purified by nickel affinity chromatography. Phosphorylation reactions (50 μl) contained 3 μM (unphosphorylated) ERK2, 20 mM MOPS (pH 7.4), 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM MgCl_2_, 3 mM ATP containing 100 cpn/pmol γ-32P]ATP, and 10–200 μM myelin basic protein (MBP, Sigma number M-1891) (39). Reactions were initiated by the addition of the ATP mixture, incubated at room temperature for 45 min, placed on ice, and immediately terminated by adding 13 μl of 5 × SDS-PAGE sample buffer for 3 min. Reaction products were separated by SDS-PAGE and visualized by autoradiography. MBP bands were excised from gels and their radioactivity was quantified by scintillation counting. Kinetic constants were determined by graphing and fitting the data to the equation: \( y = \frac{V_{max}}{K_m + x} \), where \( y \) is the reaction velocity and \( x \) is the MBP concentration using SigmaPlot 5.0 software.
ERK2-MEK1 Interaction

**Fig. 1.** The interactions of ERK2 with MEK1, MEK2, various MEK1 and MEK2 mutants, and an ERK1/2 docking site from chicken RSK (most similar to human RSK1) examined using the yeast two-hybrid system. The yeast strain L40 was co-transformed with pGAD-ERK2 and empty pLexA vector, pLexA-MEK1, or plasmids encoding LexA fusions to the indicated proteins. Interactions were tested by streaking co-transformed yeast isolates on a medium lacking His, Leu, and Trp and observing streaks for growth. A representative plate from one of three experiments is shown. Multiple independent yeast transformants were streaked for each interaction tested. The expression of all GAD and LexA fusion proteins in yeast was confirmed by immunoblotting (data not shown).

**Immunofluorescence**—HEK293 cells were cultured on collagen-coated coverslips placed in 60-mm dishes until reaching 50% confluency. One ml of a calcium phosphate precipitate containing 4 g/ml of each plasmid DNA was added to the 5 ml of culture medium. Transfection efficiency was 20–30%. After an overnight incubation, cells were deprived of serum for 24 h, fixed in phosphate-buffered saline (PBS) containing 3.7% formaldehyde and made permeable by incubating in PBS containing 0.5% Triton X-100. Cells were incubated for 1 h at room temperature in PBS containing 1% bovine serum albumin and 40 g/ml anti-FLAG M2 antibody (Sigma number F3165). Cells were then washed and incubated for 30 min in PBS-bovine serum albumin containing a goat anti-mouse secondary antibody (Molecular Probes, Alexa 488) that was diluted 1:3000. Cells were mounted using Polymount and visualized using a Zeiss Axiocam Microscope with Open Lab Software.

**RESULTS**

**ERK2-MEK1/2 Interactions Examined Using the Yeast Two-hybrid System**—To analyze the nature of the ERK2-MEK1/2 interaction detected in two-hybrid tests, we examined the interaction of ERK2 with wild type MEK1 and MEK2, as well as with several mutant forms of these MEKs. A fusion of ERK2 to the LexA DNA-binding domain independently activated transcription of the HIS3 auxotrophy reporter gene borne by the yeast strain L40 (data not shown). For this reason, a fusion of ERK2 to the Ga14 transcriptional activation domain (GAD-ERK2) was used in all two-hybrid experiments. Although wild type MEK1 interacted only weakly with ERK2, the catalytically defective mutant MEK1-K97M interacted robustly with ERK2 in two-hybrid assays (Fig. 1). In contrast to MEK1, MEK2 interacted well with ERK2 in both the wild type and catalytically inactive forms (MEK2-K101A) (Fig. 1).

To determine whether the MEK1 D domain (residues 33–93) is required for interaction with ERK2 in two-hybrid assays, we examined the interaction of ERK2 with a MEK1 mutant lacking the N-terminal 32 amino acids (MEK1-(33–93)) (16, 21, 40). ERK2 did not interact with MEK1-(33–93), but did interact with the catalytically active mutant MEK1-(33–93)-K97M (Fig. 1), indicating that the MEK1 D domain is not required for a two-hybrid interaction with ERK2.

Interactions between ERK2 and several isolated D domain sequences were also examined. ERK2 did not interact with the D domain-containing fragment of MEK1 (residues 1–32, Fig. 1). This result is consistent with our finding that the MEK1 D domain is not required for the two-hybrid interaction of ERK2 and MEK1-K97M. However, ERK2 did associate with a previously characterized 64-residue docking site from the C terminus of ribosomal S6 kinase (RSK-(689–752), Fig. 1), which contains a D domain at its extreme C terminus (41, 42). In keeping with previous findings, deletion of the D domain sequence from this construct (RSK-(689–738)) eliminated the interaction with ERK2 (Fig. 1) (41). These two-hybrid tests suggest significant differences in the affinity of ERK2 for various D domain sequences.

To assess the importance of the activation state of MEK to the MEK-ERK interaction, we examined the two-hybrid interaction of ERK2 with constitutively active MEKs. ERK2 failed to interact with the constitutively active MEK mutants MEK1-R4F and MEK2-R4F (40) (Fig. 1). This result was expected based on suggestions in the literature that stable MEK-ERK complexes are not formed when ERK2 is phosphorylated on Tyr185 (17).

These data indicate that the MEK1 D domain is not required for a two-hybrid interaction between ERK2 and MEK1-K97M. In addition, they suggest that the strength of the ERK2-MEK1 interaction is increased when MEK1 is catalytically inactive, perhaps due to a lack of phosphorylation of ERK2 on Tyr185 (17).

**Identification of ERK2 Mutants That Fail to Bind MEK1, but Retain Other Interactions**—Several regions of MAP kinases have been implicated in interactions with MEKs (11–14, 16, 18–22). The CD domain appears to function as a general docking element, and may be utilized by the many D domain-containing proteins that interact with MAP kinases. To identify novel elements involved in ERK2-MEK1 binding, we performed an unbiased mutagenesis and two-hybrid screening protocol that identifies point mutations in ERK2 that selectively impair the interaction with MEK1.

An ERK2 mutant two-hybrid library was screened to identify mutants that had lost the ability to interact with MEK1-K97M, yet retained the ability to interact with MAP kinase-interacting kinase 1 (MK1), an ERK2 substrate (see “Experimental Procedures” for details of library and screen). Further analysis was performed on ERK2 mutants containing a single amino acid change that was not expected to have adverse effects on ERK2 kinase activity.

Based on their reduced activation in HEK293 cells (see below), four ERK2 mutants were analyzed in detail: H230R, N236K, Y261N, and S264P. The two-hybrid interactions of these mutants with MEK1-K97M, MEK2, MNK1, RSK-(689–752), and MAP kinase phosphatase 3 (MKP3) were tested to determine whether interactions with proteins other than MEK1 were affected. The mutant H230R failed to interact with MEK1-K97M, but retained the ability to interact with MEK2 in a weakened fashion (Fig. 2 and Table I). The mutant N236K, upon re-testing, was found to have only a modest defect in its interaction with MEK1-K97M (not apparent in Fig. 2), but exhibited a dramatically impaired interaction with MEK2 (Fig. 2 and Table I). The mutant N236K, upon re-testing, was found to have only a modest defect in its interaction with MEK1-K97M (not apparent in Fig. 2), but exhibited a dramatically impaired interaction with MEK2 (Fig. 2 and Table I). This result was surprising as N236K was isolated from our screen as a mutant that had lost binding to MEK1-K97M. This discrepancy may result from the fact that while the two-hybrid assays of the screen procedure were performed in diploid yeast, the two-hybrid tests shown in Fig. 2 were carried out in haploid yeast. ERK2-N236K interacted with MNK1 in a manner indistinguishable from that of wild type ERK2, and displayed only slightly weakened inter-
The mutants Y261N and S264P failed to interact with both MEK1-K97M and MEK2, but displayed nearly wild type interactions with MNK1, MKP3, and RSK-(689–752) (Fig. 2 and Table I).

The Locations of MEK1/2-specific Mutations within the ERK2 Structure—We examined the positions of the mutated residues within the three-dimensional structure of unphosphorylated ERK2 (44) and found they are clustered on the surface of the C-terminal domain near the ERK2 activation loop, either in a turn immediately preceding α-helix G (H230R), within α-helix G (N236K), or within the MAP kinase insert (Y261N and S264P) (Fig. 3, B–D). Possible implications of the locations of these residues within the ERK2 structure are discussed below.

We examined the roles of the ERK2 CD domain (residues 316–319) and MAP kinase insert sequences in the two-hybrid interactions under investigation. The ERK2 double mutant D316A/D319A failed to interact with MNK1, MKP3, and RSK-(689–752). However, ERK2-D316A/D319A retained the ability to interact with both MEK1-K97M and MEK2, albeit in a weakened fashion (Fig. 2 and Table I). These findings are
magnified in B

Asp319 play important roles in the interaction of ERK2 with Asn236, Tyr261, and Ser264 is as in mutated in ERK2 mutants with selective loss of interaction with MEK1/2.

The activation loop residues are colored black consistent with previous work indicating that Asp316 and Asp319 play important roles in the interaction of ERK2 with a number of proteins that contain D domain sequences. Deletion of the MAP kinase insert from ERK2 (ERK2-Δ242–271) yielded a protein that did not interact detectably with MEK1–K97M or MEK2 (Fig. 2 and Table I). ERK2-Δ242–271 displayed an apparent wild type interaction with MNK1, but reduced interactions with MKP3 and RSK-(689–752) (Fig. 2 and Table I). These data suggest that the MAP kinase insert sequence plays a critical role in the association of ERK2 with both of its activators (MEK1 and MEK2). On the other hand, stable associations between ERK2 and the substrates MNK1 and RSK-(689–752) and the phosphatase MKP3 do not require the presence of the insert sequence.

**Mutant ERK2 Proteins Impaired in MEK1 Binding Are Inefficiently Activated by MEK1/2 in Mammalian Cells**—We investigated whether the described ERK2 mutant proteins, which were defective in their interactions with MEKS in two-hybrid experiments, were activated by endogenous MEKS in cells. To test this, mutant ERK2 cDNAs were subcloned into a mammalian expression vector and expressed in HEK293 cells. Three copies of the FLAG epitope were inserted at the N terminus of ERK2, yielding a protein that migrates with a molecular weight of 46,000 by SDS-PAGE (Fig. 4A). Following a period of serum starvation, transfected cells were treated with EGF, lysed, and the resulting protein extracts were analyzed by immunoblotting with antibodies specific for doubly phosphorylated (active) ERK1/2. ERK2 mutants H230R and Y261N exhibited dramatically reduced levels of dual phosphorylation following EGF treatment, indicative of a defect in their activation (Fig. 4A). Mutants N236K and S264P consistently displayed modestly reduced levels of dual phosphorylation in response to EGF treatment (Fig. 4A), indicating a less severe defect in activation. ERK2 proteins containing MEK1/2-specific mutations also showed reduced levels of kinase activity following EGF stimulation, confirming that the lower levels of dual phosphorylation reflected reduced activity (Fig. 4A, bottom panel). As expected from the above blots, mutants H230R and Y261N displayed the lowest activities (Fig. 4A, bottom panel). These data, together with the two-hybrid results above, sug-

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**Table I**

| ERK2 protein | Interaction partner | Vector | MEK1-K97M | MEK2 | MNK1 | MKP3 | RSK-(689–752) |
|--------------|---------------------|--------|-----------|------|------|------|----------------|
| Wild type    |                     | 0.015  | 0.176     | 0.350| 0.614| 0.962| 1.941          |
| D316A–D319A  |                     | 0.027  | 0.115     | 0.098| 0.002| 0    | 0              |
| Δ242–271     |                     | 0.031  | 0.032     | 0.017| 0.754| 0.106| 1.146          |
| H230R        |                     | 0.040  | 0.038     | 0.077| 0.681| 0.187| 1.550          |
| N236K        |                     | 0.037  | 0.105     | 0.035| 0.661| 0.880| 1.406          |
| Y261N        |                     | 0.046  | 0.048     | 0.030| 0.556| 0.756| 1.801          |
| S264P        |                     | 0.055  | 0.052     | 0.033| 0.894| 0.617| 1.604          |

**Fig. 3.** The locations of MEK1/2–specific point mutations within the three-dimensional structure of unphosphorylated ERK2 (44). A, a schematic representation of ERK2 drawn using Molscript 2.1.2 (45). α-Helices are colored red and the β-strands are colored blue. The activation loop (residues 173–187) is colored red and the MAP kinase insert (residues 243–273) is colored magenta. The proposed D domain-binding site (CD domain (21)) is indicated with an arrowhead and the text “CD.” A dashed line square indicates the portion of the figure magnified in B. The α-carbon and side chain atoms of residues His230, Asn236, Tyr261, and Ser264 are drawn as ball-and-stick. B, a magnification of the indicated portion of A. The α-carbon and side chain atoms of residues His230, Asn236, Tyr261, and Ser264 are drawn as ball-and-stick with the following color scheme: carbon (black), nitrogen (blue), and oxygen (red). C, a space-filling model of a portion of ERK2 rendered using Insight II 2000 software (Molecular Simulations). Activation loop residues are colored red. The color scheme for the individual atoms of residues His230, Asn236, Tyr261, and Ser264 is as in B except that carbon atoms are colored green. D, the sequence of residues 222 to 292 of rat ERK2 with secondary structural elements indicated as in (44) and colored as in A. Individual residues highlighted with red (His230, Asn236, Tyr261, and Ser264) are those mutated in ERK2 mutants with selective loss of interaction with MEK1/2.
suggest that the mutated ERK2 proteins have impaired interactions with MEK1/2, and that this property inhibits their activation in HEK293 cells following EGFR stimulation.

**MEK1 and MEK2 Are Activated to Similar Extents by EGF Treatment of HEK293 Cells**—We compared the activation of endogenous MEK1 and MEK2 by EGF in HEK293 cells. MEK1 and MEK2 were immunoprecipitated from EGF-treated HEK293 cell lysates and their activities were examined in immune complex kinase assays. Antibodies selective for MEK1 and MEK2 have been described (37) and are directed against non-conserved sequences in the MEK1/2 proline-rich insert. As seen in Fig. 4, B and C, EGF treatment stimulated MEK1 and MEK2 activity to a similar extent. This finding suggests that the varying degrees of EGF-stimulated activation of the ERK2 mutants described above is unlikely to result from different degrees of activation of MEK1 and MEK2.

**Defects in Activation of ERK2 Mutants Are Overcome if Constitutively Active MEK1 Is Overexpressed**—Impaired activation of the ERK2 mutants (Fig. 4A) might result from a reduced ERK2 affinity for MEK1/2. If so, a significant increase in the intracellular abundance of MEK1/2 might overcome this defect. To test this hypothesis, HEK293 cells were transiently co-transfected with constitutively active MEK1-R4F and either wild type ERK2 or the various ERK2 mutants. In response to co-expression of MEK1-R4F, all four ERK2 mutants showed levels of dual-phosphorylation comparable with that of wild type ERK2 (Fig. 5). When the MEK1-R4F-stimulated kinase activities of the mutant proteins were examined, ERK2 mutants H230R and N236K were found to have activities similar to that of wild type ERK2 (Fig. 5). ERK2 mutants Y261N and S264P, although activated, showed somewhat reduced activities toward substrate (Fig. 5). These data indicate that the overexpression of constitutively active MEK1 largely overcomes the defects in the activation of the ERK2 mutants that were observed following EGF treatment. Therefore, once activated in mammalian cells, these mutant proteins are similar to wild type ERK2 in their kinase activity, indicating that structural changes caused by the mutations are not significantly altering their function.

**Kinetic Analysis of ERK2 Proteins Containing MEK1/2-specific Mutations**—To assess quantitatively the impact of the mutations H230R, N236K, Y261N, and S264P on ERK2 func-
We performed in vitro kinase assays using recombinant proteins. These mutant proteins, as well as wild type ERK2 and catalytically defective ERK2-K52R were expressed in E. coli and purified to near homogeneity (Fig. 6). The basal activity of these recombinant proteins toward the model substrate MBP was examined. The concentration of MBP was varied from 10 to 200 μM and steady state kinetic constants were determined (Table II). The apparent $K_{\text{m,app}}$ (MBP) (11.7 ± 2.4 μM) and $k_{\text{cat,app}}$ (0.025 ± 0.0009 min$^{-1}$) values determined for wild type, unphosphorylated ERK2 were similar to those reported by Prowse and Lew (39) ($K_{\text{m}}$ 50 ± 10 μM; $k_{\text{cat}}$ 0.012 ± 0.009 min$^{-1}$). The H230R and Y261N mutant proteins, which were the most dramatically altered, both showed an ∼3.5-fold increase in the apparent $K_{\text{m,app}}$ for MBP (Table II) and displayed decreases in the apparent $k_{\text{cat,app}}$ of 3.4- and 1.9-fold, respectively (Table II). The kinetic behaviors of the mutants N236K and S264P were very similar to that of wild type ERK2 (Table II). Under the assay conditions used, kinase activity associated with the ERK2-K52R protein was not detected.

**ERK2 Proteins Containing MEK1/2-specific Mutations Are Localized to the Nuclei of Transfected Cells**—Transient overexpression of ERK2 results in a predominantly nuclear localization of the transfected protein (20, 22). If exogenous MEK1 is co-expressed, the transfected ERK2 protein is largely localized to the cytoplasm of serum-starved cells. MEK1 contains a nuclear export sequence (47), and is thought to form a complex with unphosphorylated ERK2 and promote its nuclear export. The ERK2 mutants described here, being specifically impaired in their ability to interact with MEK1/2, might be insensitive to MEK1-mediated cytoplasmic localization. To test this, HEK293 cells were transfected with MEK1 and FLAG-tagged versions of either wild type ERK2 or the various ERK2 mutants. Following serum starvation, cells were fixed, immunostained for the FLAG epitope, and stained with diamidino-2-phenylindole dihydrochloride to identify nuclei. As seen in Fig. 7, the co-expression of MEK1 resulted in a largely cytoplasmic or perinuclear localization of transfected wild type ERK2. However, in the presence of co-transfected MEK1, ERK2 mutants H230R, N236K, Y261N, and S264P consistently showed a predominantly nuclear staining pattern that was similar to that of ERK2 expressed without MEK1 (Fig. 7). The mutants Y261N and S264P were consistently more dramatically localized to the nucleus than were the mutants H230R and N236K. We conclude that these ERK2 mutants interact poorly with MEK1 in HEK293 cells, resulting in their having a predominantly nuclear staining profile even in the presence of overexpressed MEK1.

**DISCUSSION**

Fidelity in signal transduction often results from the intrinsic specificities of pathway components for one another (48). This may be the case for the activation of MAP kinases by...
MEKs, as the MEK-MAP kinase specificity observed in vitro largely mirrors that observed in vivo. The molecular details of MEK-MAP kinase specificity are being pursued experimentally. However, because crystal structures of MEKs and MEK-MAP kinase complexes are not available, their interactions have been studied by mutagenesis. Investigators have created chimeras and other variants that lose interaction or have altered specificities. The results of many such studies have suggested that multiple structural elements of ERK2, including the N terminus, α-helix C, the activation loop, the MAP kinase insert, and the C-terminal L16 segment, are important for interaction with MEKs.

In this study, we identified ERK2 point mutants that fail to interact with MEK1 in a two-hybrid screen and subsequently examined their properties in mammalian cells. In two-hybrid experiments, these mutant ERK2 proteins retain the ability to interact with MNK1, MKP3, and a docking site from RSK. Because the latter three proteins bind to ERK2 through D domains (21, 41, 42), the novel mutations we describe here are unlikely to impair ERK2 binding to D domains. Consistent with this notion, the MEK1/2-specific mutations we describe occur in ERK2 residues well removed from the putative D domain-binding site (CD domain) (see Fig. 3A). Thus, these mutations appear to interfere selectively with the binding of ERK2 to MEK1/2. Although the MEK1/2-specific mutations described here are located on the same surface of ERK2 as the active site, they have little or no adverse effect on protein kinase activity. This was true both when these proteins were immunoprecipitated from mammalian cells expressing constitutively active MEK1 (Fig. 5) and when their kinetic behavior was examined in vitro (Table II). Interestingly, the residue comparable with His230 was mutated in a dominant gain-of-function allele of the MAP kinase Fus3p (D227N) (49).

The analysis of these mutants in mammalian cells is complicated by the fact that other proteins may influence the association of ERK2 with MEK1/2. Scaffolds such as yeast Ste5p promote the formation of cascade complexes that may control accessibility and localization of cascade components. However, since the mutants described here were not exported from the nucleus in the presence of MEK1 (Fig. 7), their association with MEK1 appears to be weak despite scaffolding or other protein-protein interactions that may take place.

The three-dimensional structures of both inactive and active ERK2 have been determined by x-ray crystallography (44, 50). As with all protein kinases, ERK2 consists of a small N-terminal domain, made up largely of β-strands, and a larger, primarily α-helical C-terminal domain (see Fig. 3A). The active site is formed at the interface of the folding domains. In the crystal structure of unphosphorylated ERK2, the activation loop extends from the active site and folds down upon the C-terminal domain, making contacts with residues from both the N terminus of α-helix G and the MAP kinase insert (44) (see Fig. 3, B and C). Upon phosphorylation, this loop refolds, losing earlier interactions and forming new contacts with both domains (50). Structural features unique to MAP kinases are the above mentioned insert of about 30 residues between kinase subdomains X and XI and a 45-residue C-terminal extension referred to as loop 16 (L16) (44). In MAP kinases, the C-terminal L16 extension winds back over the N-terminal domain such that the N and C termini are close together (44) (see Fig. 3A). Insertions into the kinase core between subdomains X and XI are found only in MAP kinases, cyclin-dependent kinases, and glycogen-synthase kinase 3 (GSK3). However, in cyclin-dependent kinases 2 and 6 this segment adopts a conformation distinct from the two α-helices observed in MAP kinases (51–53).

Two of the mutations identified in this screen (Y261N and S264P) occur at residues within the MAP kinase insert, which forms contacts with the activation loop in unphosphorylated ERK2 (44) (see Fig. 3). The other two residues, His230 and Asn236, precede or are in α-helix G, which is in close contact with the insert. Biochemical or biological functions of the MAP kinase insert have not previously been determined. Our results implicate recognition of ERK2 by MEKs.

As crystal structures of MEK-MAP kinase complexes are not available, it is difficult to predict how the mutations described here might impair MEK1-ERK2 complex formation. One or more of the residues His230, Asn236, Tyr261, or Ser264 might directly contact MEK1 in a MEK1-ERK2 complex. Alternatively, the described mutations might act indirectly, by altering the conformations of other ERK2 residues critical for MEK1 binding. MEK1 usually phosphorylates ERK2 on Tyr185 prior to phosphorylating Thr183 (43, 54). Because the side chain of Tyr185 is buried in unphosphorylated ERK2 (44), a conformational change in the activation loop is required for the hydroxyl of Tyr185 to enter the active site of MEK1. The binding of MEK1 to unphosphorylated ERK2 may drive this conformational change. The close proximity of the mutated residues His230 and Tyr261 to residues making contacts with the activation loop (Tyr231, Leu232, and Ala252) raises the possibility that the mutations H230R and Y261N might indirectly alter the behavior of the flexible activation loop in unphosphorylated ERK2 (see Fig. 3, B and C). Alterations in the conformation or flexibility of this loop might result in impaired MEK1 binding. Wolf et al. (19) have demonstrated that mutations in the ERK2 activation loop (residues 176–178 to Ala) impair association with MEK1 in vivo. Consistent with this finding, the ERK2 double mutant T183E/Y185E, which has a disordered activation loop in crystals (46), exhibits an impaired interaction with MEK1-K97M in two-hybrid experiments.2 Hopefully, high-resolution crystal structures of MEK-MAP kinase complexes will be obtained in the future. Such structures should provide substantial insight into how MEK-MAP kinase signaling specificity is achieved and may better explain how specific mutations disrupt MEK-MAP kinase recognition.

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