Constitutively active mutant forms of signaling enzymes provide insight into mechanisms of activation as well as useful molecular tools for probing downstream targets. In this study, point mutations in ERK2 at conserved residues L73P and S151D were identified that individually led to 8–12-fold increased specific activity and in combination reached 50-fold, indicating synergistic interactions between these residues. Examination by mass spectrometry, phosphatase sensitivity, and Western blotting revealed that the mutations enhanced ERK2 activity by facilitating intramolecular autophosphorylation predominantly at Tyr-185 and to a lesser extent at Thr-183 and that phosphorylation at both sites is required for activation. A set of short molecular dynamics simulations were carried out using different random seeds to sample locally accessible configurations. Simulations of the active mutant showed potential hydrogen bonding interactions between the phosphoryl acceptor and catalytic nucleophile, which could account for enhanced intramolecular autophosphorylation. In intact cells, the ERK2 mutants were functionally active in phosphorylating Elk-1 and RSK1 and activating the c-fos promoter. This activity was only partially reduced upon treatment of cells with the MKK1/2 inhibitor, U0126, indicating that in vivo the mechanism of ERK2 activation occurs substantially through autophosphorylation and partially through phosphorylation by MKK1/2.

Mitogen-activated protein (MAP) kinase enzymes are key components in signaling pathways essential for cellular responses to extracellular stimuli such as growth factors or cellular stress (for review, see Ref. 1). Constitutively active, gain-of-function (GOF), oncogenic, and dominant negative mutants of components in MAP kinase pathways have served as useful tools to probe pathway epistasis and to identify specific signaling targets and mechanisms of regulation. For example, a constitutively active MAP kinase kinase (MKK1) mutant, created by changing regulatory phosphorylation sites to acidic residues (S218D, S222D), has been used to demonstrate sufficiency of MKK1/ERK signaling in cell transformation and differentiation (2–4). Such mutations mimic activation by phosphorylation, minimizing variations in substrate specificity and kinetic parameters compared with MKK1 wild type (5). In contrast, active mutants of mammalian MAP kinases with properties similar to their physiological diphosphorylated forms have proven more difficult to design. Introducing acidic amino acid substitutions at phosphorylation sites in mammalian extracellular signal-regulated kinase, ERK2, failed to enhance basal activity and in fact suppressed activity relative to wild type (6, 7).

Recently, constitutive activation of ERK2 was accomplished by forming a chimera with its upstream activator MKK1 (8). The resulting fusion was activated through a mechanism involving dual phosphorylation of ERK2, facilitated by its proximity to MKK1. However, the ERK2-MKK1 fusion is more than twice the size of monomeric ERK2 and shows differences in nuclear translocation behavior after overexpression. Furthermore, it has recently been shown that several acidic residues in ERK2 (312–320) are involved in binding MKK1 as well as substrates containing D domain interaction motifs (9–12). Thus, the presence of MKK1 could potentially interfere with substrate-ERK2 interactions. Active forms of MAP kinases involving more conservative mutations are desirable for investigating physiological responses to pathway signaling.

In this study, we report point mutations that in combination enhance basal-specific activity of ERK2 by 50-fold in vitro as well as induce GOF behavior in cell models for signaling. These mutations significantly enhanced the rate of ERK2 intramolecular autophosphorylation at Thr-183 and Tyr-185 in the absence of MKK1/2. Such constructs provide new insight into mechanisms of MAP kinase regulation as well as provide important molecular tools for probing signal transduction mechanisms in intact cell systems.

EXPERIMENTAL PROCEDURES


text continues...
the luciferase gene in pGL3 LUC. A herpes simplex virus thymidine kinase promoter directing constitutive expression of Renilla luciferase was used to control for transfection efficiency and cell number in luciferase transfection experiments (pRL-TK, Promega). pCMV5 Myc–ERK2-MKK1-LA and pCMV5L HA-Elk-1 were gifts of Melanie Cobb (8), and pCMV5L HA-ERK2-MKK1-LA was a gift of Rony Dalviy, University of Texas, Austin, TX (13).

Preparation of Recombinant Protein—NgT7–5 rat His6–ERK2 was transformed into BL21(DE3)-pLysS Escherichia coli, and bacteria were induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside as previously described (6). Cells were harvested by centrifugation and frozen at −80 °C. Wild type ERK2 and ERK2-K52R were phosphorylated to 1.7 mol/mol stoichiometry with recombinant MKK1-78 (AN/S218E/M219D/N221D/S222D) and purified as described (14).

Enzyme Assays—Autophosphorylation of recombinant ERK2 was carried out by incubating 2–4 μg of purified recombinant ERK2 for 2 h at 30 °C in assay buffer (25 mM Tris-HCl, pH 8, 2 mM dithiothreitol, 0.1 mM ATP, 10 mM MgCl2, 2.5 μCi of [γ-32P]ATP, and 5 μg of rabbit myelin basic protein (Sigma). Reaction products were separated by SDS-PAGE (12.5% acrylamide, 0.083% bisacrylamide) or mass spectrometry. Kinase activity assays (25 μl) were carried out by incubating recombinant ERK2 (0.5 μg) or diphosphorylated wild type ERK2 (0.1 μg) at 30 °C in the presence of assay buffer containing 2.5 μCi of [γ-32P]ATP and 5 μg of goat myelin basic protein (Sigma). Reactions were carried out for 1 or 10 min, quenched with Laemmli sample buffer, separated by SDS-PAGE, and quantified by PhosphoImager analysis (Molecular Dynamics). Phosphatase assays were carried out by incubating 1 μM of recombinant ERK2 with 0.2 μg of purified PP2A(A) (15) (a generous gift of Marc Mumby, University of Texas Southwestern) and 8 units of CD45 (Calbiochem) for 30 min at 30 °C in 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol in the presence or absence of okadaic acid plus microcystin (2 μg each) or sodium orthovanadate (VO4−3) (2 mM). Phosphatase assays were stopped by adding okadaic acid/microcystin to PP2A(A) or VO4−3 or CD45, and kinase activities were measured for 1 min at 30 °C using myelin basic protein as a substrate.

Mass Spectrometry—Mass spectrometry analysis was performed as described (16, 17). Briefly, ~10 μg of purified recombinant ERK2 was digested with 1 μg of trypsin in 0.2 mM Tris-HCl, pH 8, 1 mM CaCl2, 1.6 μM dithiothreitol and 50 mM NaCl in assay buffer prepared by reverse phase capillary high performance liquid chromatography (POROS R120) in 0.1% formic acid with 0–80% acetonitrile gradient and analyzed by LC/MS and LC/MS/MS (PE Sciex API-III+, PerkinElmer Life Sciences).

Cell Culture and Transfections—HEK 293 cells were grown in 6-well plates in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Life Technologies, Inc.). Cells were transiently transfected at 40%–50% confluence using LipofectAMINE PLUS™ (Life Technologies, Inc.) for 3 h, placed in fresh Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum in the presence or absence of 20 μM U0126 (Promega), and harvested 20–24 h later by the addition of passive lysis buffer (Promega). Transfections for luciferase assays included 0.75 μg of ERK2, MKK1, or ERK2-MKK1-LA cDNA constructs and 0.25 μg of FoxO-luciferase reporter. 0.25 μg of pRL-TK plasmid was added to each transfection reaction to normalize for cell number and transfection efficiency. Firefly and Renilla luciferase activities were measured in lysates using the dual luciferase assay kit (Promega) and a MicroLumat LB96P luminometer (EG&G Berthold). Firefly luciferase activity was normalized to the Renilla luciferase activity and reported as relative luciferase activity units. Transfections for immunoblotting included 0.75 μg of ERK2, MKK1, or ERK2-MKK1-LA and 0.75 μg of RSK1 or Elk-1 cDNA constructs.

Immunoblotting—Recombinant ERK2 protein (0.1 μg) or cell lysate was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PerkinElmer Life Sciences) Antibodies used to probe blots were rabbit αERK2 (1:2000) (C-14, Santa Cruz), which recognizes the C terminus of recombinant ERK2 and is overlapped by mouse αERK1 (1:2000) (gift of Rony Segel), which, respectively, recognize the monophospho-Thr-183 and monophospho-Tyr-185 form of ERK1/2; mouse αPTEY/ERK1/2 (1:2000) (Sigma), which recognizes the doubly phosphorylated form of ERK1/2; mouse αHA (1:2000) (12CA5, Berkeley Antibody Co.), which was used to examine HA-GRK1, HA-Elk-1, and HA-MKK1 expression; rabbit α-phospho-GRK1 (ThrP)-360/SerP)-364 (1:1000) (Cell Signaling Technology), which recognizes GRK1 phosphorylated at Thr-360 and Ser-364; and rabbit α-phospho-Elk-1 SerP)-339 (1:1000) (Signal Transduction Laboratories), which recognizes Elk-1 phosphorylated at Ser339.

Molecular Dynamics Simulations—The mutant molecule was designed by using Leu-73 with proline and Ser-151 with aspartic acid in the unphosphorylated ERK2 crystallographic model (Protein Data Bank code: 1erK) using the Biopolymer module in INSIGHT II 97.0 (Molecular Simulations, Inc.). Mutant and wild type molecules were minimized using 50 steps of steepest descent followed by 450 steps of conjugate-gradient minimization without solvent and counterions in AMBER5 (18, 19). The molecules were then equilibrated over 2,000 steps from 100 to 298 K using different initial randomized velocities for each run. Each run number for the mutant and wild type molecules employed identical initial randomizations (see Table I). In vacuo simulations were then carried out for 10,000 steps, and the coordinates of each simulation were analyzed at 1,000-step intervals. The equilibration and dynamics simulations were carried out on the Cray T3E supercomputer at the San Diego Supercomputer Center on four parallel processors. Each run lasted 210 min.

RESULTS

Point Mutations Lead to Constitutive Activation of ERK2—Several point mutations have been shown to activate MAP kinases in genetic systems. Brkl et al. (21) describe three dominant GOF mutations in Saccharomycyes cerevisiae FUS3 that correspond to residues D48N, I161L, and D227N and three recessive GOF mutations that correspond to residues Y7H, I9K, and C28Y (22). A dominant GOF mutation in FUS3, L63P, was identified in a screen for mutants resistant to growth repression in high salt (23). The sevenmaker mutation, D334N, was identified in the Drosophila melanogaster MAP kinase, rol2 (24), and has been reported to disrupt interactions between MAP kinases and phosphatases, thus elevating kinase activity (25, 26). A mutation at K136Q was identified within a peptide from mouse ERK2 expressed as a tumor rejection antigen in a carcinogen-induced BALB/c tumor (27), suggesting a role of this mutation in maintaining the malignant phenotype. Although the behavior of each mutant suggests involvement in pathway activation, most have never been tested for their direct effects on enzyme-specific activity. Only the sevenmaker mutation has been examined and shows no effect on basal-specific activity of recombinant ERK2 (25). Finally, a mutation, S194D, was discovered by alignment in which the conserved sequence DLKPSEN in MKK1 was modified to resemble the sequence DLKPSN in MKK1 was modified to resemble the sequence DLKPSN in AMP-dependent protein kinase. This mutant resulted in a 15-fold enhancement of MKK1 activity.3 All 10 residues described above are conserved in rat ERK2 and correspond to E58N, V171L, H230N, F17H, V19K, C38Y, L73P, D319N, K136Q, and S151D (28). A mutation at K136Q was identified within a peptide from mouse ERK2 expressed as a tumor rejection antigen in a carcinogen-induced BALB/c tumor (27), suggesting a role of this mutation in maintaining the malignant phenotype. Although the behavior of each mutant suggests involvement in pathway activation, most have never been tested for their direct effects on enzyme-specific activity. Only the sevenmaker mutation has been examined and shows no effect on basal-specific activity of recombinant ERK2 (25). Finally, a mutation, S194D, was discovered by alignment in which the conserved sequence DLKPSEN in MKK1 was modified to resemble the sequence DLKPSN in AMP-dependent protein kinase. This mutant resulted in a 15-fold enhancement of MKK1 activity.3 All 10 residues described above are conserved in rat ERK2 and correspond to E58N, V171L, H230N, F17H, V19K, C38Y, L73P, D319N, K136Q, and S151D. Their location in the x-ray structure of unphosphorylated, inactive ERK2 is shown in Fig. 1.

Each of these mutations was incorporated singly and in combination into rat ERK2 by site-directed mutagenesis. The mutants were expressed in E. coli and purified, and specific activity was measured by incorporation of phosphate into myelin basic protein (Fig. 2). Single mutations at L73P and S151D showed increases in basal-specific activity reaching 8–12-fold over wild type. C38Y and D319N showed increases of 2–5-fold, and F17H, V19K, E58N, K136Q, V171L, and H230N showed no measurable increase. Double mutant S151D/D319N and L73P/D319N enhanced activities by 30- and 50-fold, respectively, which represent a greater than additive synergy. Combining all three mutations L73P/S151D/D319N yielded nearly a 100-fold enhancement over wild type.
increased activity over wild type, the highest specific activity achieved in these experiments. Adding C38Y, E58N, or K136Q in combination with these mutations (C38Y/L73P/S151D, E58N/S151D/D319N, L73P/K136Q/S151D) resulted in only modest effects on activity (0.8–1.6-fold). Consistent inhibition was observed upon incorporating V171L, H230N, or the combination of C38Y and K136Q. In summary, of 10 mutations, significant constitutive activation of ERK2 in vitro was obtained with L73P, S151D, and D319N.

**Activating ERK2 Mutations Cause Enhanced Intramolecular Autophosphorylation**—Wild type ERK2 autophosphorylates weakly at Thr-183 and Tyr-185 through an intramolecular reaction resulting in a small elevation of basal activity (6, 28, 29). Mono- and diphosphorylated forms of wild type ERK2

comigrate on SDS-PAGE but resolve from the unphosphorylated form of ERK2 and can be monitored by gel mobility retardation (30) (Fig. 3A). Likewise, ERK2 mutants migrated in two forms. Under basal conditions, the mutants modified at C38Y, L73P, or S151D exhibited increased abundance of the slower migrating form compared with wild type ERK2 (Fig. 3A). This increase resulted from autophosphorylation, because in vitro incubation of each mutant with Mg$^{2+}$-ATP elevated the proportion of gel-shifted forms, whereas catalytically inactive mutant ERK2-K52R was not gel-shifted. Incorporation of D319N increased the overall gel mobility of ERK2 compared with wild type, as previously reported (25).

Autophosphorylation of ERK2 was examined by Western analysis using antibodies specific to monophosphorylated (Thr(P)-183 or Tyr(P)-185) or diphosphorylated (Thr(P)-183 + Tyr(P)-185) ERK (Fig. 3B). Increased reactivity of a gel-shifted form with opY-ERK occurred with all active mutant forms (L73P, S151D, S151D/D319N, L73P/S151D, and L73P/S151D/D319N) in the absence of ATP. Increased opY-ERK reactivity of all forms of ERK2 except K52R was observed upon incubation with Mg$^{2+}$-ATP. Although reactivity of opT-ERK was also observed, the reactive band corresponded to the faster-migrating form. This indicates nonspecific reactivity of this antibody with unphosphorylated ERK2, which is confirmed by its reactivity with ERK2-K52R. Because no reactivity of this antibody was observed with the slower migrating, phosphorylated form of ERK2, monothreonyl-phosphorylated ERK2 is below the detection limit of this antibody. Reactivity of L73P, S151D, S151D/D319N, L73P/S151D, and L73P/S151D/D319N with antibody selective for diphosphorylated ERK (pTy-ERK) suggested that L73P and S151D facilitate diphosphorylation. This reactivity also increased upon incubation with Mg$^{2+}$-ATP. These results suggest that L73P and S151D preferentially enhance autophosphorylation at Tyr-185 and increase autophosphorylation at Thr-183 to a small degree. The relative level of auto-diphosphorylation of L73P, S151D, S151D/D319N, L73P/S151D, and L73P/S151D/D319N correlated with their observed increased *in vitro* activities.

We next addressed whether autophosphorylation occurred through an intramolecular or intermolecular mechanism by examining the ability of ERK2-L73P/S151D/D319N to autophosphorylate ERK2-K52R in trans. This took advantage of the behavior of phosphorylated ERK2-L73P/S151D/D319N, which migrates faster than phosphorylated ERK2-K52R (Fig. 3C). Mixing increasing amounts of ERK2-L73P/S151D/D319N with a fixed amount of ERK2-K52R in the presence of Mg$^{2+}$-[γ-32P]ATP resulted in $^{32}$P incorporation into ERK2-L73P/S151D/D319N but not ERK2-K52R. This indicates that trans-autophosphorylation does not occur and that the mechanism of phosphoryl transfer is therefore intramolecular.

A mutation at T183E has been reported to elevate ERK2 activity 100-fold after Tyr-185 phosphorylation by MKK1 compared with basal wild type ERK2 activity (6). We therefore tested the effects of combining L73P and S151D mutations with T183E in hopes of further enhancing basal activity. However, neither enhanced activity nor enhanced autophosphorylation was observed in any mutant combination, and in fact incorporation of T183E reduced the specific activities of L73P, L73P/S151D, and L73P/S151D/D319N by 3–6-fold (data not shown).

Phosphorylation of ERK2-L73P/S151D was examined by LC/MS and MS/MS by monitoring the total ion current of MH$^+$ and MH$^{+1}$ corresponding to the tryptic peptide VADPDHHGITGLT183EY185VATR. Wild type ERK2 was ≥98% unphosphorylated at Thr-183 and Tyr-185 (Fig. 4A) but after a 2-h incubation with Mg$^{2+}$-ATP became monophosphorylated to 10% mol/mol stoichiometry. The monophosphorylated peptide eluted just before the unphosphorylated form,
indicating phosphorylation at Tyr-185 as previously characterized (17). In contrast, ERK2-L73P/S151D was 90\% mono-phosphorylated at Tyr-185 and 10\% unphosphorylated. No ions were observed corresponding to mono-threonyl-phosphorylated peptide, which elutes just after the unphosphorylated form (17). MS/MS sequencing of the MH2 ion (742.6 Da) was performed, confirming mono-tyrosyl phosphorylation of the L73P/S151D mutant (Fig. 4B). Ions corresponding to mono-threonyl-phosphorylated peptide were not detected by MS/MS. Diphosphorylated peptides were negligible even after incubation with Mg\(^{2+}\)-ATP for both wild type and ERK2-L73P/S151D (Fig. 4A). Thus, the major effect of mutagenesis is to enhance autophosphorylation at Tyr-185.

**Fig. 3.** Elevated autophosphorylation rates in active ERK2 mutants occurs through an intramolecular mechanism. Recombinant purified wild type and mutant ERK2 were incubated in the presence or absence of Mg\(^{2+}\)-ATP for 2 h at 30°C and examined by SDS-PAGE followed by Coomassie staining (A) or Western blot analysis probed with phospho-specific ERK1/2 antibodies (pY-ERK, pT-ERK, or pTpY-ERK) or αC-terminal ERK2 (B). pERK denotes migration of mono- or diphosphorylated ERK2, ERK denotes migration of unphosphorylated ERK2, αpT-ERK reactivity corresponds to the faster migrating, unphosphorylated ERK, whereas αpY-ERK reactivity corresponds to the slower migrating phosphorylated ERK form. C, recombinant purified ERK2-L73P/S151D/D319N and ERK2-K52R were incubated alone or mixed together in the presence of Mg\(^{2+}\)-[γ-\(^{32}\)P]ATP for 2 h at 30°C. In lanes 1 and 4–7, ERK2-K52R was present at 0.75 μg. In lanes 3–7, ERK2-L73P/S151D/D319N was present at 0.75, 0.004, 0.04, 0.2, and 0.75 μg. In lanes 1 and 8, ERK2-K52R phosphorylated by active MKK1 (pK52R) was present at 0.75 μg.

**Mutant ERK2 Is Activated by Autophosphorylation at Thr-183 and Tyr-185.—**The evidence above did not conclusively resolve whether the enhanced specific activity of the ERK2 mutants (Fig. 2) was caused by diphosphorylation at both Thr-183 and Tyr-185 or monophosphorylation at Tyr-185 in combination with the point mutations. LC/MS may not be sensitive enough to reveal low amounts of diphosphorylated ERK2 since the threshold of detection is ~1–2% mol/mol under the conditions used; therefore, diphosphorylation of ERK2 could not be ruled out. On the other hand, reactivity with αpTpY-ERK antibodies does not unambiguously prove diposphorylation due to potential cross-reactivity with monophosphorylated pY-ERK.
To assess the importance of threonine versus tyrosine phosphorylation toward activation by the L73P and S151D point mutations, we examined sensitivity to inactivation by phosphatases in the presence of PP2A(AC) and CD45, which selectively dephosphorylate serine/threonine and tyrosine, respectively. As expected, loss of activity was observed when phosphorylated wild type ERK2 was incubated with either PP2A(AC) or CD45 (Fig. 5A). Under these conditions PP2A(AC) and CD45 selectively dephosphorylate Thr(P)-183 and Tyr(P)-185, respectively, as confirmed by LC/MS (data not shown). Treatment of ERK2-L73P/S151D with either phosphatase also resulted in loss of activity, indicating that autophosphorylation...
at both Thr-183 and Tyr-185 is required for enhanced activity. Phosphorylated wild type ERK2 showed no shift to a faster-migrating form upon treatment with either phosphatase (Fig. 5B); this reflects the high degree of diphosphorylation of the preparation and confirms specificity of dephosphorylation by each phosphatase. On the other hand, treatment of ERK2-L73P/S151D with CD45 resulted in increased gel mobility, indicating that most of the mutant protein is mono-tyrosine-phosphorylated (Fig. 5B), in agreement with Western and LC/MS analysis (Fig. 3B and 4A). However, increased mobility was not observed with PP2A(AC), indicating that the loss of mutant ERK2 activity reflects dephosphorylation of phosphothreonine not dephosphorylation of the mono-tyrosine-phosphorylated form.

Comparison of Wild Type Versus Mutant ERK2 by Molecular Dynamics—To examine the potential basis of point mutations on ERK2 structure, L73P and S151D substitutions were incorporated into the x-ray structure of unphosphorylated ERK2 by homology modeling using INSIGHT II. Wild type and mutant structures were energy-minimized and then subjected to molecular dynamic simulations for 20 ps in vacuo using AMBER5. Twelve simulations were run in parallel for wild type ERK2 and ERK2-L73P/S151D, each run using a different initial-randomized velocity applied to both forms of ERK. A set of short runs with different random seeds enabled sampling of locally accessible configuration. Conformers were then analyzed for distance relationships between selected residues. For each run, 10 conformers were averaged over 2-ps intervals up to 20 ps.

The most striking differences were observed in distances between the hydroxyl oxygen atom of Tyr-185 and the δ oxygen of the catalytic residue Asp-147. In 2 of 12 independent runs, the distances between the phospho-acceptor hydroxyl and closest δ oxygen were within 3.5 Å in the L73P/S151D mutant; this was 4.6 Å shorter than the corresponding distance in wild type ERK2 (Table I). A comparison of wild type versus mutant structures in run 5 is shown in Fig. 6. The simulation suggests that the L73P/S151D mutant has the ability to adopt conformers that bring the phospho-acceptor oxygen in Tyr-185 within hydrogen-bonding distance of the catalytic nucleophile. In contrast, Tyr-185 in wild type ERK2 is located more than 8 Å away from Asp-147 and is buried in a hydrophobic pocket and hydrogen-bonded to the guanidinium group of Arg-146. No significant differences were observed in distances between the Thr-183 phospho-acceptor oxygen and Asp-147 nucleophilic oxygen in wild type versus mutant ERK2 (Table I).

ERK2 Mutants Are Functionally Active in Intact Cells—Next we evaluated the behavior of the active ERK2 mutants transfected into mammalian cells with respect to their ability to phosphorylate known physiological targets of ERK2. ERK2 phosphorylates the transcription factor Elk-1, which when complexed with serum response factor binds the serum response promoter element and enhances transcription from the c-fos promoter. A c-fos-luciferase reporter was cotransfected into HEK 293 cells with empty vector or wild type or mutant...
Oxygen-oxygen distances between Tyr-185 or Tyr-183 and Asp-147 in wild type ERK2 versus ERK2-L73P/S151D

| Run | Wild type ERK2 | ERK2-L73P/S151D | Wild type ERK2 | ERK2-L73P/S151D |
|-----|----------------|-----------------|----------------|-----------------|
| 1   | 9.1 (0.4)      | 9.5 (1.1)       | 16.4 (0.5)     | 15.7 (0.7)      |
| 2   | 8.6 (1.2)      | 7.1 (0.6)       | 16.1 (0.4)     | 14.7 (0.9)      |
| 3   | 9.2 (0.7)      | 9.7 (0.5)       | 15.4 (0.6)     | 14.3 (0.5)      |
| 4   | 9.1 (0.4)      | 9.8 (0.8)       | 16.2 (0.5)     | 15.6 (0.3)      |
| 5   | 8.4 (0.8)      | 2.7 (0.2)       | 16.2 (0.4)     | 16.2 (0.5)      |
| 6   | 8.1 (0.6)      | 3.5 (1.7)       | 16.4 (0.7)     | 15.0 (0.8)      |
| 7   | 8.5 (0.7)      | 9.7 (0.5)       | 15.6 (0.4)     | 15.7 (0.5)      |
| 8   | 7.9 (0.5)      | 9.5 (0.8)       | 16.0 (0.6)     | 15.1 (0.7)      |
| 9   | 12.0 (1.4)     | 14.1 (0.4)      | 14.2 (1.1)     | 12.3 (0.9)      |
| 10  | 8.8 (0.6)      | 10.0 (0.7)      | 15.5 (0.8)     | 13.8 (1.0)      |
| 11  | 10.2 (0.9)     | 11.9 (1.2)      | 15.9 (0.5)     | 16.8 (0.9)      |
| 12  | 7.8 (0.7)      | 7.7 (1.1)       | 13.3 (0.4)     | 15.1 (0.5)      |

*Values indicate oxygen-oxygen distances in Å between hydroxyl atoms in Tyr-185 or Thr-183 and the nearest δ oxygen in Asp-147. Values are reported for 12 runs as averages (S.D.) for 10 conformers in each run, sampled at 2- to 20-ps intervals between 2-20 ps.

**Distances are statistically significant between wild type and mutant ERK2; < 0.001 by t test.**

**Fig. 6. Conformers of wild type versus mutant ERK2 after molecular dynamics simulation.** Wild type ERK2 and ERK2-L73P/S151D were energy-minimized and subjected to molecular dynamics simulations in vacuo for 20 ps using AMBER 5. Conformations of wild type ERK2 (A) and ERK2-L73P/S151D (B) after 20 ps are shown, in both cases corresponding to results of run 5 in Table I. Positions of Asp-147 and Tyr-185 side chains are shown indicating distances between the phospho-acceptor oxygen of Tyr-185 and the nearest δ oxygen in Asp-147. Hydrogen-bonding distances between the phospho-acceptor oxygen and base nucleophile were observed in ERK2-L73P/S151D in 2 of 12 simulations but were never observed in wild type ERK2.

ERK2, and luciferase induction was measured (Fig. 7A). Parallel assays compared luciferase induction in response to constitutively active mutants MKK1-R4F, MKK1-G1C, and ERK2-MKK1-LA. Western analysis shows comparable expression of recombinant ERK2 mutants in these experiments (Fig. 7B).

The single point mutations L73P, S151D, D319N showed little increase in transcriptional activation compared with wild type ERK2. However, ERK2-L73P/S151D exhibited a 10-fold increase in luciferase induction (calculated after subtraction of empty vector control). This was comparable with the observed 8- and 12-fold activation by MKK1-R4F and MKK1-G1C, respectively, and greater than the 3-fold activation by ERK2-MKK1-LA. In contrast, ERK2-L73P/S151D/D319N exhibited only 6-fold activation. This was surprising considering that its in vitro activity was 2 times greater than ERK2-L73P/S151D but could be accounted for by recent evidence showing that acidic residues within ERK2 (312–320) are important for substrate binding (9–12). C38Y, K136Q, C38Y/L73P/S151D, C38Y/L73P/S151D, L73P/K136Q/S151D, C38Y/L73P/K136Q/S151D, and C38Y/L73P/K136Q/S151D/D319N ERK2 mutants were also examined but showed no enhancement of activity above ERK2-L73P/S151D and ERK2-L73P/S151D/D319N (data not shown). Correlated responses were observed when Elk-1 phosphorylation was measured after cotransfection of HA-Elk-1 with ERK2-MKK1-LA, wild type ERK2, or mutant ERK2. Phosphorylation of Elk-1 was monitored by its reactivity with a phospho-specific antibody recognizing pS383, a known ERK2 phosphorylation site. Although single mutations in ERK2 had minimal effect on Elk-1 phosphorylation, ERK2-L73P/S151D increased phosphorylation levels comparable with ERK2-MKK1-LA (Fig. 8A). Western analysis revealed that both ERK2-MKK1-LA and ERK2-L73P/S151D retarded the gel mobility of Elk-1 to multiple forms, most likely because of multiple ERK2 phosphorylation sites on Elk-1. Western blots also demonstrated comparable expression of ERK2 constructs (Fig. 8A).

Further assays examined HA-RSK1 as a substrate for mutant ERK2 when cotransfected into HEK 293 cells. Phosphorylation of RSK1 was monitored using a phospho-specific antibody recognizing Thr(P)-360 and pS362 on RSK1, both sites that are directly targeted by ERK2. ERK2-L73P/S151D induced phosphorylation of RSK1 comparable with constitutively active ERK2-MKK1-LA (Fig. 8B). ERK2-L73P/S151D and ERK2-L73P/S151D/D319N mutants also enhanced RSK1 phosphorylation although to a lesser extent than ERK2-L73P/S151D. Similar results were obtained in COS-1 cells (data not shown). Like Elk-1, RSK1 showed gel mobility-shifted forms, consistent with multiple ERK2 phosphorylation sites. The increased RSK1 phosphorylation responsive to ERK2-L73P/S151D correlated with a 15-fold increase in its activity toward S6 peptide in kinase assays measured with immunoprecipitated RSK1 (data not shown), demonstrating that mutant ERK2 enhances activation as well as phosphorylation of RSK1.

ERK2-L73P/S151D Activity Is Resistant to Cell-permeable Inhibitors of MKK1—Finally, we examined the resistance of mutant ERK2 activity to inhibition by U0126, a cell-permeable MKK1/2 inhibitor (Fig. 9A). Assays measuring activation of the c-fos-luciferase reporter showed that both MKK1-G1C and ERK2-MKK1-LA were strongly inhibited by U0126, as expected from the ability of this compound to directly bind and inhibit MKK1/2 (31). In contrast, luciferase activity in cells

**TABLE I**

| Run | Wild type ERK2 | ERK2-L73P/S151D | Wild type ERK2 | ERK2-L73P/S151D |
|-----|----------------|-----------------|----------------|-----------------|
| 1   | 9.1 (0.4)      | 9.5 (1.1)       | 16.4 (0.5)     | 15.7 (0.7)      |
| 2   | 8.6 (1.2)      | 7.1 (0.6)       | 16.1 (0.4)     | 14.7 (0.9)      |
| 3   | 9.2 (0.7)      | 9.7 (0.5)       | 15.4 (0.6)     | 14.3 (0.5)      |
| 4   | 9.1 (0.4)      | 9.8 (0.8)       | 16.2 (0.5)     | 15.6 (0.3)      |
| 5   | 8.4 (0.8)      | 2.7 (0.2)       | 16.2 (0.4)     | 16.2 (0.5)      |
| 6   | 8.1 (0.6)      | 3.5 (1.7)       | 16.4 (0.7)     | 15.0 (0.8)      |
| 7   | 8.5 (0.7)      | 9.7 (0.5)       | 15.6 (0.4)     | 15.7 (0.5)      |
| 8   | 7.9 (0.5)      | 9.5 (0.8)       | 16.0 (0.6)     | 15.1 (0.7)      |
| 9   | 12.0 (1.4)     | 14.1 (0.4)      | 14.2 (1.1)     | 12.3 (0.9)      |
| 10  | 8.8 (0.6)      | 10.0 (0.7)      | 15.5 (0.8)     | 13.8 (1.0)      |
| 11  | 10.2 (0.9)     | 11.9 (1.2)      | 15.9 (0.5)     | 16.8 (0.9)      |
| 12  | 7.8 (0.7)      | 7.7 (1.1)       | 13.3 (0.4)     | 15.1 (0.5)      |
transfected with ERK2-L73P/S151D, although partially suppressed by U0126, still enhanced activity compared with cells transfected with wild type ERK2. ERK2-L73P/S151D also retained partial activity with respect to phosphorylation and gel mobility retardation of RSK1, which was completely abolished for MKK1-G1C and ERK2-MKK1-LA in the presence of U0126 (Fig. 9B). Under these conditions, the phosphorylation state of ERK2 was examined using αH9251pTpY-ERK antibody. Phosphorylation of ERK2-MKK1-LA or endogenous ERK1/2 stimulated by MKK1-G1C was completely suppressed by U0126, whereas ERK2-L73P/S151D and ERK2-L73P/S151D/D319N retained significant phosphorylation in the presence of inhibitor. These results demonstrate that the active point mutants of ERK2 are partially regulated by endogenous MKK1/2 but still retain properties of enhanced autophosphorylation, allowing them to function independently of upstream activator.

DISCUSSION

We have created a novel, constitutively active mammalian ERK2 by combining point mutations that act synergistically, independent of upstream activation. In vitro ERK2-L73P/S151D and ERK2-L73P/S151D/D319N exhibit 50–100-fold enhanced basal-specific activity over wild type ERK2, which correlates with an enhanced rate of autophosphorylation. ERK2-L73P/S151D and ERK2-L73P/S151D/D319N retain increased phosphorylation under physiological conditions when expressed in HEK 293 cells. ERK2-L73P/S151D phosphorylates and activates the known ERK1/2 targets Elk-1 and RSK1 to similar levels as constitutively active MKK1 and ERK2-MKK1-LA. We have also shown that ERK2-L73P/S151D retains its ability to activate the c-fos promoter and phosphorylate RSK1 in the presence of the MKK1/2 inhibitor U0126, indicating that these point mutations are constitutively activated in the absence of upstream regulators. Thus, these mutants functionally enhance MAP kinase pathway signaling at the level of ERK and will be useful for identifying downstream ERK2 targets without complicating effects of upstream pathway components.

The combined evidence indicates that these point mutations activate ERK2 by enhancing autophosphorylation through a cis-regulated mechanism. Autophosphorylation at Tyr-185 is predominant over Thr-183; however, phosphorylation of both residues is required for enzyme activation. This contrasts the mechanism of activation of the ERK2-MKK1-LA fusion protein, which is also dependent on dual phosphorylation of ERK2 at Thr-183 and Tyr-185 but involves catalysis by the MKK1 fusion domain (8). Because the ERK2-MKK1-LA fusion cannot be recombinantly produced in bacteria without proteolytic degradation, we were unable to compare specific activities of our mutants to the fusion protein in vitro. However, ERK2-L73P/S151D increases Elk-1 and RSK1 phosphorylation and activation to levels comparable with constitutively activated MKK1 and ERK2-MKK1-LA after expression in mammalian cells. Western analysis reveals that the level of diphosphorylated ERK2-L73P/S151D is similar to that of endogenous ERK1/2 induced upon expression of MKK1-G1C, explaining the simi-
...larities in magnitude of cellular signaling.

The behavior of the ERK2 mutants suggests that enzyme activation occurs by relieving structural constraints in the catalytic site that suppress intramolecular autophosphorylation and autoactivation. The x-ray structure of ERK2 in its inactive conformation shows Tyr-185 pointed toward the catalytic cleft in a buried solvent-inaccessible position located 8 Å away from Asp-147. Asp-147 functions as the proton acceptor for base-assisted catalysis, increasing the nucleophilicity of the substrate for phosphoryl transfer. Conceivably, S151D and L73P mutations may facilitate intramolecular autophosphorylation by indirectly altering the position of the phosphate acceptor with respect to catalytic residues. This is supported by molecular dynamics simulations showing the ability of the mutant to adopt conformers that enable hydrogen bonding distances between the phosphoryl acceptor oxygen on Tyr-185 and the δ oxygen within Asp-147. Ser-151 interacts with residues that contact the activation lip as well as Asp-147 and...
Asp-165, the latter of which functions in metal coordination. It is possible that incorporation of a negatively charged residue at position 151 leads to changes in flexibility of the activation lip and/or reorientation of key catalytic residues to favor autophosphorylation. Examination of the x-ray structure also reveals that Leu-73 within helix C forms hydrophobic interactions with Phe-166 within the sequence DFG. Proline insertion into helix C could conceivably facilitate autophosphorylation by altering Leu-73–Phe-166 interactions, leading to subtle repositioning of Asp-165, although such changes were not apparent in the 20-ps simulations. Structural studies will be needed to more conclusively address mechanisms underlying facilitated intramolecular autophosphorylation and enzyme activation.

The D319N mutation is conserved in MAP kinases and is known to direct binding interactions between kinase and the docking domain of protein substrates or MAP kinase phos-

**Fig. 9.** ERK2-L73P/S151D signals in the absence of MKK1/2 activity. **A**, HEK 293 cells were cotransfected with c-fos-luciferase reporter and ERK2 or MKK1 expression constructs as in Fig. 4. After transfection, cells were incubated in the presence or absence of 20 μM U0126 for 24 h before harvesting. Values are reported as averages and S.D. in triplicate measurements of firefly luciferase activities normalized to Renilla luciferase activities. **B**, cells were cotransfected with HA-RSK1 and ERK2 or MKK1 as in Fig. 5B and incubated in the presence or absence of 20 μM U0126 for 24 h before harvest. Cell lysate (15 μg) was analyzed by Western blotting probed with phospho-specific antibody to RSK1 recognizing Thr(P)-380 and Ser(P)-364.
Activation of ERK2 by Synergistic Point Mutations

We observed a 2-fold decrease in transcriptional signaling upon incorporation of this mutation, suggesting that the regulation of ERK2 activity by phosphatases may not be as high in HEK 293 cells as in other systems. We hypothesize that the D319N mutation, indicating that autoactivation by reducing interactions with upstream activators.

References

1. Lewis, T. S., Shapiro, P. S., and Ahn, N. G. (1998) Adv. Cancer Res. 74, 49–139
2. Brunet, A., Pages, G., and Pouyssegur, J. (1994) Oncogene 9, 3579–3587
3. Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994) Cell 77, 841–852
4. Mansour, S. J., Matzen, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994) Science 265, 966–970
5. Mansour, S. J., Candia, J. M., Matsuura, J. E., Manning, M. C., and Ahn, N. G. (1996) Biochemistry 35, 15529–15536
6. Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Ebert, D., Geppert, T. D., and Cobb, M. H. (1993) J. Biol. Chem. 268, 5097–5106
7. Zhang, J., Zhang, F., Ebert, D., Cobb, M. H., and Goldsmith, E. J. (1995) Structure (London) 3, 299–307
8. Robinson, M. J., Stoppec, S. A., Goldsmith, E., White, M. A., and Cobb, M. H. (1998) Curr. Biol. 8, 1141–1150
9. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U., and Arkinstall, S. (1998) Science 280, 1262–1265
10. Rubinfeld, H., Hanoch, T., and Seger, R. (1999) J. Biol. Chem. 274, 30349–30352
11. Tanoue, T., Adachi, M., Moriguchi, T., and Nishida, E. (2000) Nat. Cell Biol. 2, 110–116
12. Nichols, A., Camps, M., Gillieron, C., Chabert, C., Brunet, A., Wilsbacher, J., Cobb, M., Pouyssegur, J., Shaw, J. P., and Arkinstall, S. (2000) J. Biol. Chem. 275, 24413–24421
13. Dalby, K. N., Morence, N., Caudwell, F. B., Avruch, J., and Cohen, P. (1998) J. Biol. Chem. 273, 1496–1505
14. Shapiro, P. S., Vaisberg, E., Hunt, A. J., Tolwinski, N. S., Whalen, A. M., McIntosh, J. R., and Ahn, N. G. (1998) J. Cell Biol. 142, 1535–1545
15. Mumbey, M. C., Russell, K. L., Garrard, L. J., and Green, D. D. (1987) J. Biol. Chem. 262, 6257–6265
16. Resing, K. A., Mansour, S. J., Hermann, A. S., Johnson, R. S., Candia, J. M., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1996) Biochemistry 34, 2610–2620
17. Resing, K. A., and Ahn, N. G. (1997) Methods Enzymol. 293, 29–44
18. Cornell, W. D., Cieplak, P., Bayly, C. I., I., R., G., Merz, K. M. J., Perusco, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) J. Am. Chem. Soc. 117, 5179–5197
19. Case, D. A., Pearlman, D. A., Caldwell, J. W., Cheatham, T. E., III, Ross, W. S., Simmerling, C., Darden, T., Merz, K. M., Stanton, R. V., Cheng, A. Vincent, J. J., Crowley, M., Ferguson, D. M., Radmer, R., Seibel, G. L., Singh, U. C., Weiner, P., and Kollman, P. A. (1997) AMBER 5.0 (San Francisco, CA, UCSF).
20. Ryckaert, J. P., Cicotti, G., and Berendsen, H. J. C. (1977) Journal of Computational Physics 23, 327–336
21. Brill, J. A., Elion, E. A., and Fink, G. R. (1996) Mol. Cell. Biol. 5, 297–312
22. Cobb, M. H., and Goldsmith, E. J. (1995) J. Biol. Chem. 270, 14843–14846
23. Hall, J. P., Cherkasova, Y., Elion, E., Gustin, M. C., and Winter, E. (1996) Mol. Cell. Biol. 16, 6715–6723
24. Brunner, D., Oellers, N., Szabad, J., Biggs, W. H., and Zipursky, S. L., and Hafen, E. (1996) Cell 80, 875–885
25. Bott, C. M., Thornecroft, S. G., and Marshall, C. J. (1994) FEBS Lett. 352, 201–205
26. Ikeda, H., Ohta, N., Furukawa, K., Miyazaki, H., Wang, L., Kuribayashi, K., Old, L. J., and Shiku, H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6375–6379
27. Seger, R., Ahn, N. G., Boulton, T. G., Yancopoulos, G. D., Panayotatos, N., Radziejewska, E., Ericsson, L., Bratlien, R. L., Cobb, M. H., and Krebs, E. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 6142–6146
28. Posada, J. W., Ue, J., Michel, H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5778–5783
29. Posada, J., Sanghera, J., Pelech, S., Aebersold, R., and Cooper, J. J. (1991) Mol. Cell. Biol. 11, 2517–2528
30. Favata, M. F., Horwich, R. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Freer, W. S., Van Dyk, D. E., Pitta, W. J., Earl, B. A., Hobbs, F., Copeland, R. A., Magolda, R. L., Scherle, P. A., and Trzaskos, J. M. (1998) J. Biol. Chem. 273, 18623–18632
31. Zhang, F., Strand, A., Robbins, D., Cobb, M. H., and Goldsmith, E. J. (1994) Nature 370, 704–711
32. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
33. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sec. D 50, 869–873
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